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BLOOD PARASITE DETECTION AND BoLA-DQA1 GENETIC DIVERSITY IN CATTLE FROM TUNISIA

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Abstract

*Blood parasites, particularly those of the Anaplasma marginale, Babesia and Theileria spp., present a challenge to successful livestock farming. In the present work, PCR analysis was carried out to detect possible infection by the above-mentioned parasites in three cattle populations from northern Tunisia. We also sequenced exon 2 of the BoLA-DQA1 gene in 17 Holstein cattle. Our results showed a low level of infection by the screened parasites, with prevalences of 8.8%, 5.9% and 0.0% for Theileria spp., Anaplasma mariginale and Babesia ssp., respectively. On the other hand, a total of 11 alleles were observed in the BoLA-DQA1 gene in the analysed samples. Six alleles were detected for the first time. BoLA-DQA1*10011 and *0101 alleles were the most frequent. These two alleles were also the most frequent in all Holstein cattle populations so far studied. Finally, four and eight amino acid positions were under positive selection by DATAMONKEY and PAML, respectively. Such selection, associated with high polymorphism observed in the BoLA-DQA1 gene, might suggest an important qualitative and quantitative parasite pressure that would favour distinct allele types.*

Key words*: Anaplasma, Babesia, BoLA-DQA1, cattle, Theileria.*

INTRODUCTION

The livestock production sector plays an important role in Tunisia's economy with a contribution of approximately 40% of the value of agricultural products (Belguesmi, 2023). However, this sector in general and that of cattle farming in particular is facing several constraints with diseases (parasites, viruses, and bacteria) constitute one of the biggest problems. Among these diseases, those transmitted by ticks limit the growth of animal farming sector and affect health and productivity of animals in various regions of the world (de Castro, 1997). Bovine theileriosis, babesiosis and anaplasmosis are considered among the most economically important diseases. Additionally, animals that recover from an acute infection may become long-term carriers without the infection being detected microscopically (Brown, 1990). Indeed, blood smear microscopy is often the preferred diagnostic method for these parasites (Bono et al., 2008). To overcome this problem, conventional PCR assays can be used effectively for the specific detection of several species of piroplasms and *A. marginale* (Almeria et al., 2001).

On the other hand, as the diversity of the MHC loci reflects adaptive and non-adaptive evolutionary processes within and between populations, it is of great interest to a wide range of scientists, including breeders, population geneticists, and evolutionary biologists (Goszczynski et al., 2014; Takeshima et al., 2014). One of the most significant factors affecting genetic diversity is the domestication bottleneck experienced by most domesticated animals (Zhang et al., 2013). This bottleneck reduces genetic diversity compared to their wild ancestors and alters the distribution of genetic variation among loci (Buckler et al., 2001). However, for MHC genes, while reduced variability might be the outcome of population bottlenecks, a high level of diversity could could result from balancing selection driven by pathogens or other mechanisms despite extreme population bottlenecks (see Bohórquez et al. (2020) for an overview).

The MHC in cattle (also known as bovine leukocyte antigen-BoLA) is located on chromosome 23 and is similar to other mammals' MHC (Takeshima & Aida, 2006). Previous studies of MHC genes in cattle have detected a significant association of the genetic diversity of these genes with certain diseases, such as mastitis (Takeshima et al., 2008; Yoshida et al., 2012), leukemia (Zanotti et al., 1990), ketosis (Mejdell et al., 1994) and infection by ectoparasites (Martinez et al., 2006). Other studies have also associated BoLA gene polymorphism with protein composition and milk fat content (Nascimento et al., 2006) as well as milk production (Rupp et al., 2007).

Here, we first used PCR technique to detect and identify blood parasite infections in cattle breeds in North Tunisia. Updating the prevalence of these parasites will allow to evaluate and to take appropriate measures to eradicate them. Second, the polymorphism of the BoLA-DQA1 gene was studied in seventeen Tunisian Holstein cattle. We used several tests to evaluate the effect of selection - as an evolutionary process - shaping DQA1 sequence diversity.

MATERIALS AND METHODS

Samples collection

Blood samples were collected randomly from 68 dairy cattle from North Tunisia between April and June 2023. The studied animals were from intensive (29 samples from Bousalem) and extensive (23 from Fernana, 16 de Menzel Bourguiba) farming. The studied cattle population belong mainly to the Holstein breed with the age of animals ranging between 1 and 10 years. The blood samples collected in 15 ml tubes containing a few drops of EDTA were immediately stored at -20°C.

DNA extraction and PCR-based blood parasite detection

DNA from whole-blood samples was extracted using the «FavorPrepTM Tissue Genomic DNA Extraction» Kit for DNA purification. All samples were controlled for successful DNA extraction using PCR amplification with the primer pairs PCO3/PCO4 that amplify the bovine β-globin gene (Konnai et al., 2006).

The detection of *Theileria* ssp., *Babesia* ssp., and *A. marginale* was performed using PCR as previously described by (Adaszek & Winiarczyk, 2008; Lew et al., 2002).

PCR and sequencing of BoLA-DQA1

Amplification of 374 bp long sequences including the whole Exon 2 of the BoLA-DQA1 gene was performed for 17 unrelated cows of Tunisian Holstein breed [Fernana (n = 6), Menzel Bourguiba (n = 6) Bousalem (n = 5)] using the primers described by Kulaj et al. (2015). The PCR products were then purified with ExoSAP enzymes and both strands were sequenced using an ABI 3130xl DNA Analyzer.

Statistical analyses of BoLA-DQA1 sequences

The obtained sequences were aligned and edited using the BioEdit v.7 program (Hall, 1999). Alleles of our DQA sequences were reconstructed with Phase 2.1.1 (Stephens et al., 2001) using five replicate runs of 1000 generations after 1000 generations of burn-in.

DnaSP program (Librado & Rozas, 2009) was used to calculated genetic diversity parameters, nucleotide diversity (π), haplotype diversity (Hd), and mean number of pairwise differences (k). The same program was also used to test for deviation from neutral evolution of BoLA-DQA1 locus by D* and F* tests of Fu and Li (1993), and Tajima's D test.

To detect positive selection on the coding BoLA-DQA1 exon 2 sequences (240 bp), we used CODEML (PAML 4 package, Yang (2007)) and the DATAMONKEY web server (http//www.datamonkey.org/) (Pond & Frost, 2005). For CODEML, we have compared model M7 (beta) against M8 (beta plus omega) using the likelihood ratio test (LRT) and used the BEB to detect codons under positive selection with a posterior probability above 95% (Yang et al., 2000). For the DATAMONKEY web server (http//www.datamonkey.org/; Pond & Frost, 2005) we used four different tests to infer codons under positive selection, Single Likelihood Ancestral Counting (SLAC), Fixed Effects Likelihood (FEL), Fast Unconstrained Bayesian AppRoximation (FUBAR) and Mixed Effects Model of Evolution (MEME) (Murrell et al., 2012, 2013).

The phylogenetic neighbour-joining tree of the currently detected BoLA-DQA1 alleles was constructed using MEGA 6.0 software (Tamura et al., 2013), including all DQA1 alleles from GenBank database that have similar length with our sequences. We used the *Ovis aries*

sequences (LN827890, OK626230) as outgroups.
Finally, we used the cited above dataset to construct a median-joining (MJ) network (Bandelt et al., 1999) using the software Network 4.2.0.1 (available at http//www.fluxus-technology.com/sharenet.htm).

RESULTS AND DISCUSSIONS

Detection of blood parasites with PCR

Among the 68 cattle samples analyzed by PCR to detect possible infection by blood parasites, only ten (14.7%) were positive, each for only one type of hemoparasite. Indeed, six samples (8.8%) were infected with *Theileria spp.* and four (5.9%) by *A. marginale*. These samples presented the specific bands of the 18s rRNA genes of the genus *Theileria* (370 bp) and msp1α of A. marginal (603 bp). The presence of parasites of the genus *Babesia* was not detected in any of the analyzed samples.
During the current study, a low prevalence was observed for blood parasites of the genus Theileria and Anaplasma in cattle from Tunisia. In addition, no infection with parasites of the genus Babesia was detected. On the contrary, previous studies have shown greater infection in various regions around the world. Indeed, the microscopic study of 278 blood samples belonging to different bovine breeds in Tunisia (M'ghirbi et al., 2008), showed that 104 samples (37.4%) were positive for different species of piroplasms. Similarly, PCR analysis of 405 cattle samples in Egypt, showing clinical signs for blood parasites, indicated that 12.66% and 24.05% were positive for Babesia and Theileria spp., respectively (Nayel et al., 2012). On the other hand, Moumouni et al. (2015) observed that 71% of the samples analyzed were positive for hemoparasites. From a methodological point of view, the use of PCR for the detection of blood parasites has already shown its effectiveness. Indeed, using microscopic analysis, fluorescent antibody testing and PCR, Nayel et al. (2012) showed the absence of significant differences in the detection power of these three methods. Additionally, Almeria et al. (2001) suggested that the use of PCR was significantly more effective in the detection of *Theileria* ssp. and *Babesia* ssp. compared to microscopic observation. The low prevalence observed during the current study might indicated good management of breeding conditions which would limit the spread of the disease or that of ticks as a vector of the studied parasites. On the other hand, the use of different treatments could effectively reduce blood parasites. However, the observed prevalences cannot be generalized for the Tunisian cattle herd; they are rather an estimate of the infection rates in the studied population.

BoLA-DQA1 polymorphism

The total size of the sequences obtained in this study is 374 bp with 28 variable positions (7.49%) of which 4 (1.1%) are singletons. The average nucleotide composition across all sequences is 31.4% T, 26.1% C, 23.7% A, and 18.8% G. This composition is observed for all individuals with rare variations of around 0.1%. The values of haplotypic diversity ($h = 0.863$), nucleotide diversity ($\pi = 0.01643$) and mean pairwise differences ($k = 6.144$) were relatively high. A total of 11 different alleles were revealed in the 17 Tunisian cow samples. Among these alleles, five have been already detected in other cattle breeds (Table 1). We identified 11 alleles in 17 cattle from Tunisia belonging to the Holstein breed, of which six alleles were detected for the first time. Although this number of alleles seems very high compared to those detected by other studies, such allelic diversity is characteristic of MHC genes (Klein et al., 1993). Indeed, while only three DQA1 alleles were observed in a population of 34 cattle in Iraq (Al-Waith et al., 2018), 15 alleles were identified in 51 samples belonging to 8 cattle breeds studied by Takeshima et al. (2007). Similarly, Kulaj et al. (2015) identified 14 alleles, including three for the first time, by analyzing 71 cattle from the Polish Holstein-Friesian breed.
On the other hand, the BoLA-DQA1*0101 allele having a frequency of 0.32 in Tunisia, showed a frequency of 0.2606 in a Holstein-Frisian population (Kulaj et al., 2015) and was also the most frequent among all alleles detected in the cattle breeds studied by Takeshima et al. (2007). The second most frequent allele in Tunisian cattle was BoLA-DQA1*10011 with a frequency of 11.8%. This allele presented a frequency of 0.3592 in the Holstein-Friesian breed (Kulaj et al., 2015) and was also the most

frequent allele in the Danish black pied breed (45%; Takeshima et al., 2007). Other studies carried out by different research teams have shown that the BoLA-DQA1*10011 and *0101 alleles are the most frequent in Holstein cattle (Takeshima et al., 2008; Miyasaka et al., 2011; Schwab et al., 2009).

Table 1. List of currently detected alleles (grey shaded) and their frequency as well as alleles retrieved from GenBank and used in our study. Breeds where alleles were detected according to Takeshima et al. (2007)

GenBank Accession N°	Allele	Frequency	Breed[1]
PP335540	BoLA-DQA1*008,04	0.088	-
PP335541	BoLA-DQA1*036,01	0.029	-
PP335542	BoLA-DQA1*001,07	0.029	-
PP335543	BoLA-DQA1*001,01,01	0.147	-
PP335544	BoLA-DQA1*001,01,02	0.088	-
PP335545	BoLA-DQA1*010,01,03	0.059	-
AB257101	BoLA-DQA1*1203	-	DR
AB257102	BoLA-DQA1*0801	-	AY x LM, JE
AB257103	BoLA-DQA1*0101	0.324	BF, DB, HF
AB257104	BoLA-DQA1*0203-1	-	BF, JE, DB
AB257105	BoLA-DQA1*0204	-	BF x AY, BF, JE,
AB257106	BoLA-DQA1*0301	-	DR, JE
AB257107	BoLA-DQA1*0401	-	BF x HE, BF
AB257108	BoLA-DQA1*0103	-	DR, JE
AB257109	BoLA-DQA1*10011	0.088	BF, DB
AB257110	BoLA-DQA1*12011	0.029	DB, BF
AB257111	BoLA-DQA1*12012	-	-
AB257112	BoLA-DQA1*1401	-	DR
AB257113	BoLA-DQA1*12021	-	BF, DR, DB, JE
AB259566	BoLA-DQA1*10012	-	BF, DR, DB
AB259567	BoLA-DQA1*0102	-	BF
AB267074	BoLA-DQA1*0203-2	0.059	BF, DB
AB362375	BoLA-DQA1*1402	0.059	HF
AB362376	BoLA-DQA1*1002	-	JB
AB362377	BoLA-DQA1*12013	-	JE

[1]AY, Ayrshire; BF, British Friesian; DB, Danish Black Pied; DR, Danish Red; HE, Hereford; HF, Holstein Friesian; JB, Japanese black; JE, Jersey; LM, Limousin.

BoLA-DQA1 selection and phylogeny

The 240-coding nucleotide of the 11 detected alleles were translated into eight amino acid sequences. Among them, only BoLA-DQA1*008,04, *036,01 and *001,07 are translated to new amino acid sequences (Figure 1). Overall, neutrality tests showed that the studied sequences were selectively neutral. In fact, the Tajima test was negative (D= -0.5746) and not significant (p>0.1). The results of the Fu and Li tests were also non-significant (D* = 0.82646; F* = 0.42724) (P > 0.1). However, Positive selection by the Datamonkey web server was observed at one (site 13), two (50, 64), and three (50, 64, 71) codons by MEME, FEL, and FUBAR, respectively (Figure 1). No positive selection was suggested with SLAC. In addition, eight codons (13, 42, 50, 51, 63, 64, 70, 71) were reported under positive selection by CODEML (Figure 1). Purifying negative selection was observed at position 4 by SLAC, FEL and FUBAR and at position 65 only by FEL.

The neighbor joining (NJ) phylogenetic tree (Figure 2) indicated that the currently detected alleles in Tunisian cattle with those downloaded from GenBank were paraphyletic and were belonging mainly to two different groups. The first one encompasses 10 of the Tunisian alleles that were divided in two subgroups. The second groups contain only allele DQA1*0203-2 and other alleles from different cattle breeds.

```
                          1       10         20         30         40         50         60         70         80
BoLA-DQA1*10011           DHIGTYGISI YHTYGPSGYY THEFDGDEEF YVDLEKRETV WRLPVFSKFT SFDPQGALRN IAIVKHNLEI VIQRSNSTAA
BoLA-DQA1*0101            ....A...NV .......... .......... .......... .......... .......... ..TA.....V L.........
BoLA-DQA1*008:04          ....A...NV ..S....... .......... .......... .........A .......... ..TA.....V L.........
BoLA-DQA1*036:01          ....A...NV ..S......F .......... .......... .........A .......... ..TA.....V L.........
BoLA-DQA1*001:07          .......... ..S....... .......... .......... .......... .......... ..TA.....V L.........
BoLA-DQA1*1402            ....A...NV ..S....... .......... .......... .........A .......... ..VG.RT..V M.R.......
BoLA-DQA1*12011           .......... .......... .......... .......... .........A T......... ..TT...... ..........
BoLA-DQA1*0203-2          ....A...NV .......... .......... .......... .N..L....R R......... ..T....... ..........
------------------------------------------------------------------------------------------------------------------
SLAC
FUBAR                                                                              *              *        *
FEL                                                                                *              *
MEME                                   *
PAML                                   *                               *       * *            **       **
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Figure 1. BoLA-DQA1 exon 2 alleles of Tunisian Holstein cattle. Asterisks at the bottom of the table indicate signals of positive selection as obtained from the different tests

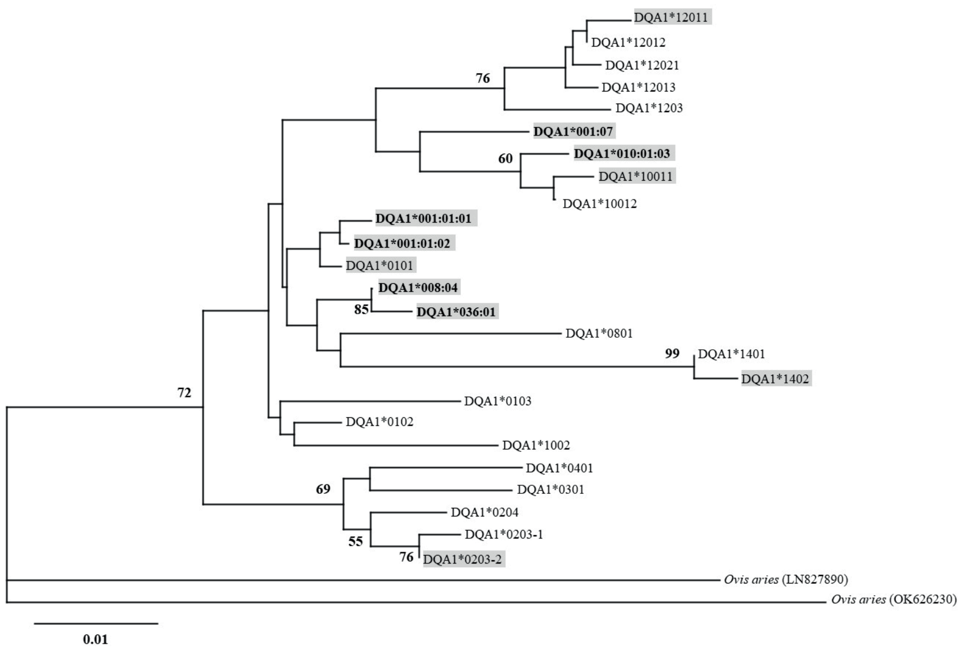

Figure 2. Neighbour-joining tree of BoLA-DQA1 allele. Numbers in the node indicate the bootstrap values if > 50%. Alleles observed in this study are grey shaded and newly identified alleles are marked in bold

The haplotype (allele) network (Figure 3) shows a central group of alleles allele that could be at the origin of the various other groups. In this central group, we find the BoLA-DQA1*0101 allele, the most frequent in Tunisia and which has also been detected in other cattle breeds. In addition, several other Tunisian alleles have a terminal position in the allele network which could indicate their relatively recent evolutionary status compared to the other central ones.

Adaptation to different pathogens is often studied by the analysis of major histocompatibility complex (MHC) class I and class II genes which are characterized by significant polymorphism in the different species analyzed so far (Klein, 1986; Weber et al., 2004; Awadi et al., 2018; Balasubramaniam et al., 2017). Our analysis of the DQA1 locus identified several codons under positive selection as evidenced from the used tests. Selection might suggest that shared alleles between different breeds can result from locally similar pathogens leading to similar natural and artificial selection pressures. Indeed, Bohórquez et al. (2020) suggested that the observed polymorphism for BoLA-DRB3 was very similar for all breeds they have studied.

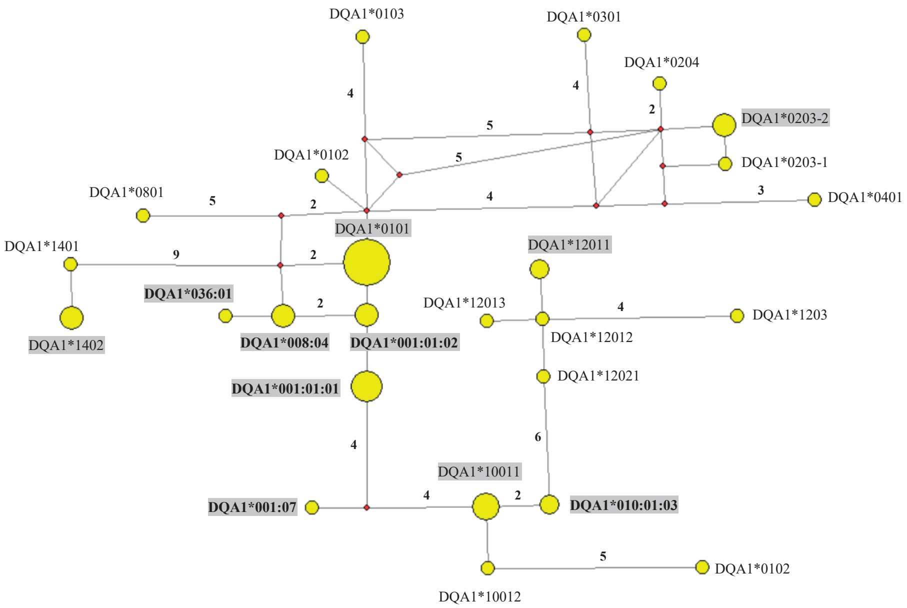

Figure 3. Median-joining network showing the relationships among BoLA-DQA1 alleles. Relative allele frequencies correspond to haplotype circle size (see Table 1). Numbers on lines connecting haplotypes indicate number of total mutation changes. Small red circle indicates inferred haplotype. Alleles observed in this study are grey shaded and newly identified alleles are marked in bold

In addition, the significant polymorphism observed in the Holstein breed in Tunisia could be linked to a significant diversity of pathogens. Indeed, the presence of seven specific alleles that have not been previously detected in different cattle populations around the world could suggest *in situ* evolution influenced by pathogens and environmental conditions. Such high polymorphism indicated that diversity in functionally important gene might persist even in the case of bottleneck events such those resulting from domestication and breeding (Bohórquez et al., 2020) In such situation, potential quantitative and qualitative differences of pathogens in varied habitats would result in high level of diversity. However, in the current study, introgression by alleles from other breeds could not be excluded.

CONCLUSIONS

The results of detection of blood parasites suggest relatively low infection rates by these parasites in the studied populations. On the contrary, the high polymorphism of the BoLA-DQA1 gene as well as the positive selection acting at several codons suggests a significant diversity of pathogens in Tunisia. On the other hand, the occurrence of a significant number of specific alleles of the Holstein breed in Tunisia might indicate an important potential of adaptation to local pathogens.

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AN APPROPRIATE APPROACH ON THE IMPLICATIONS OF MICROARRAY TECHNOLOGY FOR ANIMAL GENETIC RESEARCH

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Abstract

Microarray technology has emerged as a powerful tool in the field of animal genetic research, offering a comprehensive and high-throughput method for analysing the expression of thousands of genes simultaneously. This paper explores the implications of microarray technology in advancing understanding of animal genetics, focusing on its applications, challenges, and potential contributions to various aspects of genetic research. The paper begins by providing an overview of microarray technology, detailing its principles and the array of applications it offers for investigating gene expression, genetic variations, and regulatory mechanisms in animals. Furthermore, this paper addresses the challenges associated with microarray data analysis, emphasizing the importance of bioinformatics methods to extract meaningful insights from large-scale genomic datasets. This study aims to guide researchers in choosing appropriate methodologies, highlighting best practices, and fostering a deeper understanding of the implications and limitations of this technology in the context of animal genetic research. This exploration also contributes to the ongoing dialogue within the scientific community on optimizing the use of microarray technology to unlock the mysteries of animal genetics and advance the knowledge of biological systems.

***Key words**: animal research, gene expression, genetic data analysis, microarray.*

INTRODUCTION

Microarray technology has enabled the identification of sets of genes over and under-expressed in various pathologies, including breast cancer, prostate cancer, lung cancer, as well as in the dysregulation of certain physiological processes such as apoptosis induction and response to therapy, not only in humans but also in animals. Integrated analyses of multiple studies have highlighted generalities and specificities of gene expression in certain pathologies (Spielbauer et al., 2005; Aizpurua et al., 2023).

The use of microarrays in biomedical research is not limited to determining gene expression profiles; they are also used to detect CNV (copy number variation) at the whole genome level, with high resolution, down to a level of 5-10 kilobases and even down to a resolution of 200 bp in the case of high-resolution array CGH variants (HR-CGH) (Madescu et al., 2019).

DNA microarray technology represents a multiplex technology used in molecular biology and veterinary medicine studies. It has evolved from the Southern Blotting analysis method - DNA fragments are attached to a substrate and hybridized with labelled probes representing gene fragments or entire genes. The spots can be short gene fragments - Probes - used for hybridization with cDNA - Target, under very well-defined conditions. The Probe-Target complexes can be visualized and quantified based on fluorescence detection of a fluorophore - fluorescent marker attached to the target for relative quantification of nucleic acid abundance in the target sample. DNA microarray technology can be used for both the detection of single nucleotide polymorphisms (SNPs) and for the detection of DNA (comparative genomic hybridization studies) or RNA (detected as cDNA after reverse transcription) which may or may not be involved in protein translation (Bendixen et al., 2005; Gheyas et al., 2013).

Measuring gene expression levels based on cDNA is called "Gene Expression Analysis". Using traditional gene expression analysis methods, researchers can study a small number of genes per analysis. The development of new technologies allows tackling problems inaccessible through classical methods and discovering new targets for drug therapies (Singh et al., 2013).

"Microarrays", recently discovered DNA microarrays, allow researchers to analyse the expression of multiple genes rapidly and efficiently in a single experiment. They represent a major step in DNA analysis methodology and illustrate how new technologies provide "powerful tools" for research (Haley et al., 2006).

Overall, adopting an appropriate approach to the implications of microarray technology for animal genetic research involves leveraging its strengths while addressing its challenges, with a focus on advancing our understanding of animal genetics, improving breeding programs, and promoting animal welfare and sustainable agriculture (Stoughton et al., 2005; Kawecka et al., 2016; Szczerbal et al., 2021).

The aim of studying the implications of microarray technology for animal genetic research is multifaceted and encompasses several key objectives. This includes understanding genetic diversity within and between animal populations, mapping traits of interest, and discovering candidate genes associated with economically important traits such as growth, reproduction, disease resistance, and behaviour (Zhang et al., 2020). Additionally, the aim involves improving breeding programs through genomic selection and marker-assisted breeding, exploring functional genomics by elucidating gene expression patterns and molecular mechanisms underlying phenotypic variation, and conducting comparative genomic studies across different animal species to understand evolutionary relationships and species-specific adaptations. Furthermore, researchers aim to validate microarray-based findings using independent methods and integrate microarray data with other omics technologies for a more comprehensive understanding of complex biological systems. Ethical considerations, including the responsible use of animals in research and adherence to ethical guidelines and regulations, are also essential aspects of studying the implications of microarray technology for animal genetic research. Ultimately, the overarching goal is to advance our understanding of animal genetics, improve breeding programs, enhance animal health and welfare, and contribute to the sustainable management of animal populations for agricultural, conservation, and biomedical purposes (Khan et al., 2021).

MATERIALS AND METHODS

In order to reach the objectives of this study, 35 bibliographic sources from specialized literature were consulted. The main issues addressed refer to the types of microchips (microchips for the detection of changes in the level of gene expression, comparative genomic hybridization-CGH microarrays, mutation/polymorphism analysis microchips); the structure of a microarray; the basic principle of the microarray technique, respectively the study of gene expression - DNA analysis by microarray and interpretation the results obtained and also are presented the advantages of microarray technique.

The research methods used in this study were observation, analysis, and graphical interpretation of data from specialized literature.

RESULTS AND DISCUSSIONS

A microarray is a laboratory tool used to detect the expression of thousands of genes at the same time. DNA microarrays are microscope slides that are printed with thousands of tiny spots in defined positions, with each spot containing a known DNA sequence or gene. These instruments play a crucial role in animal genetic analysis, facilitating accurate identification and in-depth examination of their genetic profiles, ultimately enhancing research and breeding programs.

These microchips will be presented in detail from the point of view of their structure and the analysis technique, bringing to the attention of researchers not only the advantages of their use in genetic analysis (Ventimiglia et al., 2013; Wickramasinghe et al., 2014).

DNA Microarray - Genetic Testing of the Future

A microarray chip can be defined as a gene expression analysis test consisting of a micro membrane or glass slide, on which DNA samples from multiple genes are systematically arranged. Samples can be represented by DNA, cDNA, or oligonucleotides. The characteristics of these tools enable a systematic and comprehensive study of the genetic expression of an organism (Ashammakhi et al., 2020; Balakrishnan et al., 2022).

Three types of probes can be used to produce microchips: two are genomic and the third is "transcriptomic" (measuring mRNA levels). They differed in the type of DNA fixed in the spots (Table 1).

Microchips for detecting changes in gene expression levels

They are also known as gene expression analysis microchips (microarray expression analysis microchips or simply expression microchips). The spots on these microchips contained cDNA obtained by reverse transcription of mRNA from known normal or mutant genes. If the expression of a gene in the studied tissue is increased, more cDNA will hybridize at that point compared to the control, with fluorescence directed towards red (Chen et al., 2023).

Gene expression chips can be used for diagnosis of genetic diseases, identifying mutations in genes involved in multifactorial diseases (especially cell cycle control genes involved in the proliferation of neoplastic cells), drug development (for drug development, these microchips can be used to study whether a new drug reduces the overexpression of a gene involved, particularly in neoplastic development.

Comparative Genomic Hybridization (CGH) Microchips

Researchers have used this technique to identify gene amplifications and deletions in the genome, or to observe changes in the copy number of a gene involved in the genesis of a specific disease. These microchips target large portions of genomic DNA. The chromosomal location must be known for each target DNA spot on the chip. The hybridization mixture contained fluorescently labelled genomic DNA probes collected from both the normal and investigated tissues. Consequently, if the copy number of the studied gene increases, a larger amount of DNA extracted from the investigated tissue will hybridize with the target spots compared to a smaller amount of control DNA. As a result, the fluorescence of the spots turned red to a greater extent as the copy number increased (Figure 1).

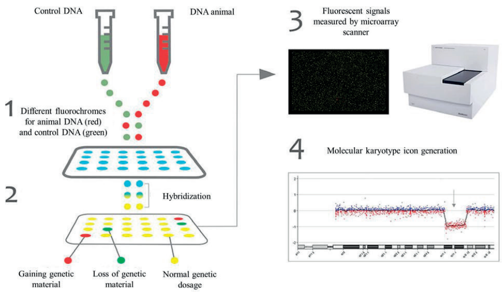

Figure 1. The basic principle of the microarray methodology (www.euroimmun.com)

The microarray CGH technique allows for the identification of Copy Number Variants (CNVs). They are classified into five categories: benign, Variants of Unknown Significance (VOUS) possibly benign, VOUS with uncertain significance, possibly pathogenic VOUS, and clearly pathogenic.

These submicroscopic genomic rearrangements are widespread throughout the genome and represent important factors in evolution, phenotypic differentiation, and susceptibility to certain conditions (Liu et al., 2015).

The advantages of the array CGH method are defined by increased clinical utility and an average detection resolution of approximately 60kb, providing a perspective on the entire genome at a high resolution, which is 10 times higher than that of classical karyotyping. It detects submicroscopic duplications and deletions, unbalanced chromosomal rearrangements, and does not require cell culture such as classical karyotyping (Naidu et al., 2012).

Mutation/Polymorphism Analysis Chips

Researchers have used these microchips to detect mutations in a single gene or single nucleotide polymorphism (SNPs).

Table 1. Types of microchips

Types of microarray	*Applications*
Microchips CGH *(comparative genomic hybridization arrays)*	tumor classification risk evaluation predicting prognosis
Expression microarrays gene *(microchips for the detection of changes in the level of gene expression)*	the development of new drugs assessment of response to medication the development of drug therapy
Analysis microchips a mutations/SNPs *(microchips for analysis of mutations or polymorphisms)*	the development of new drugs assessment of response to medication the development of drug therapy following the evolution of diseases

(data processed after Heller et al., 2002; Jenkins et al., 2002; Huang et al., 2015)

The structure of a microarray

A microarray chip consists of a small solid support onto which probes containing DNA sequences of hundreds or thousands of different genes are fixed at well-defined positions. The support usually consists of microscope slides (glass) of a standard size or can be made of silicon or polymeric membranes (nylon). DNA probes are printed in the form of spots or synthesized directly onto the support. It is crucial that the DNA probes are fixed to the support in a well-defined order, because gene identification is based on their localization in the microarray (Figure 2).

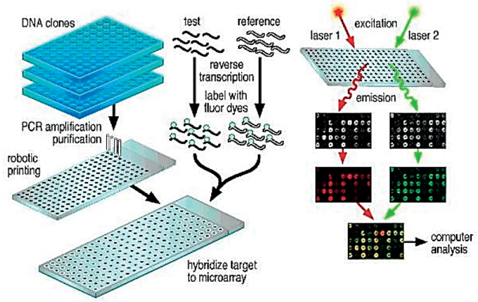

Figure 2. Structure of microarray (www.euroimmun.com)

Microarrays are miniature tests of gene fragments that are attached to glass slides. Presenting thousands of gene fragments in a single test allows the detection of gene expression changes in a significant fraction of the total genome. Linear arrays of molecules are immobilized at discrete locations on an inert surface, which allows simultaneous analysis. Microarray technology is commonly used because it is easy to implement and well controlled. A microarray is a stamp-sized glass slide that contains thousands or hundreds of thousands of spots. Each spot contains a synthetic DNA strand with a known sequence. A microarray consists of a portion (partial) of specific genes, created by placing a known DNA sequence (Nies et al., 2024).

The basic principle of the microarray technique

The basic principle of microarray technology is similar to the Southern blot (DNA-DNA hybridization) and Northern blot (RNA-DNA hybridization) techniques. It relies on the complementarity of gene sequences to recognize each other and detect the presence or absence of the DNA or RNA of interest using a series of radiological, fluorogenic, or chemiluminescent detectors (Figure 3).

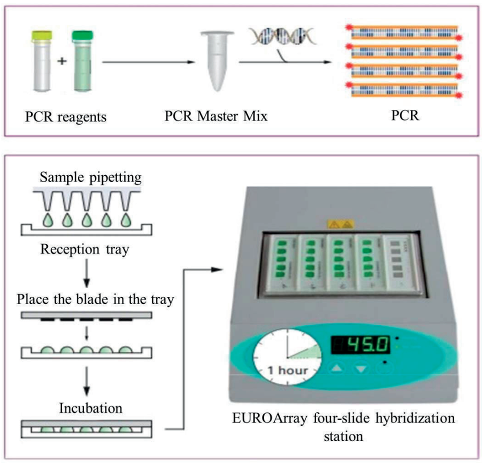

Figure 3. Microarray analyzer - EUROArrayScanner (https://microbenotes.com)

The operating principle is based on the ability of mRNA molecules to hybridize with the corresponding DNA matrix molecules. Using a microchip containing multiple DNA probes, the expression levels of hundreds or even thousands of genes in a cell can be determined in a single experiment by measuring the amount of mRNA bound to each probe on the microchip. The resulting data on the quantity of DNA bound at each spot and the gene expression profile of the cell are displayed on a computer (Figure 4).

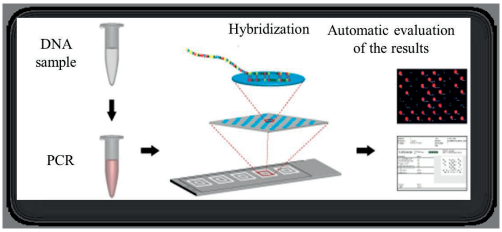

Figure 4. The operating principle of the microarray technique (https://microbenotes.com)

The first step involves isolating DNA from the blood. The DNA sequences of interest were amplified millions of times by PCR. Primers define the region to be copied, and if the DNA contains the respective sequence, the primers bind to it and amplification occurs. The samples obtained by PCR were fluorescently labelled and incubated (Pena et al., 2014).

Gene expression study - DNA analysis through the microarray technique

After hybridization, the microarray was placed in a special scanner composed of several lasers, specialized microscope, and camera. The fluorescent spots were excited by laser beams, and the microscope and camera created a digital image of the microarray. The data were stored and analysed using a computer equipped with specialized software to calculate the red/green fluorescence ratio and to analyse the intensity of each spot on the digital image of the microchip (Bao et al., 2012).

Microarray experimental setup

The hybridization step was performed with two cDNA probes for comparison (samples from diseased tissue/healthy tissue; treated cells/untreated cells) labelled with two different fluorophores (Slonim et al., 2009).

Fluorescent markers: Cy3 with an emission wavelength of 570 nm (corresponding to the green light spectrum) and Cy5 with an emission wavelength of 670 nm (corresponding to the red light spectrum) (Rajagopal et al., 2020).

The two types of probes, Cy-labeled cDNA, were mixed and hybridized on the same microarray chip, which was scanned to analyse the fluorescence signal intensity of the two fluorophores.

The relative intensities of the fluorescent signals of the two markers were analyzed as a ratio to identify genes with upregulated or downregulated expression (Figure 5).

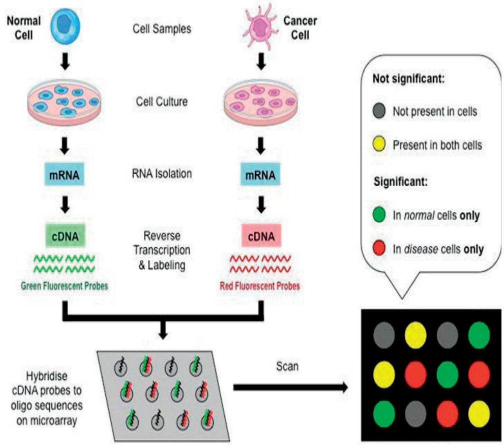

Figure 5. Experimental protocol for DNA analysis by the technique microarray (https://microbenotes.com)

Affymetrix is one of the first companies to produce microarrays, developing technology and synthesis based on combinatorial chemistry. These methods have been applied to construct high-density matrices of oligonucleotides on glass or silicon substrates (microchips) (Dufva et al., 2009).

Interpretation of the results obtained through the microarray technique

Each spot on the microchip represents a specific gene; each color represents the DNA extracted from healthy tissue (control) or the DNA sample extracted from the tissue under investigation (sample). Depending on the type of chip used, the location and intensity of each color specify the expression level (presence/absence) of a gene (or its mutation) in the DNA samples (Figure 6).

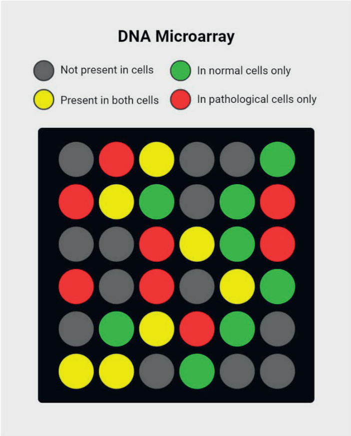

Figure 6. DNA microarray interpretation (https://microbenotes.com)

Although still in its infancy, microchip technology represents a significant first step in the field of genetics. This new technology enables scanning of the entire genome for relevant polymorphisms using gene microchips. Multiple thousands of polymorphisms can be determined simultaneously for a single subject. Currently, SNPs are selected as markers distributed throughout the genome, with the hope that functionally important polymorphisms can be associated with specific markers due to their proximity on the chromosome (Fan et al., 2010).

Such whole-genome association studies are already being used to detect susceptibility genes in a disease. Whole-genome scanning can be used similarly to determine genes involved in drug response, even when the mechanism of action of that drug is not known.

There are several advantages of microarray technology: microarrays allow for the simultaneous analysis of mRNA expression from several thousand genes; microchips can also be used to determine the gene expression pattern in a target tissue, contributing to demonstrating the mechanisms of action of a pharmacological agent in a genomic context; they can track interindividual differences in drug response in different tissues; up to 30,000 genes can be analysed at the same time; they can indicate quantitative changes in mRNA related to gene expression: during the cell cycle, during organism development from embryo to adult, after exposure to various stimuli, in pathological conditions versus normal conditions; they provide information about the functioning of a system as a whole, etc (Shinde et al., 2023; Shukla et al., 2020a; Shukla et al., 2020b).

CONCLUSIONS

Currently, microarrays are considered powerful tools in the field of genetics. This new technology allows for disease diagnosis and a better understanding of how gene expression is altered under different conditions. Furthermore, it enables the comparison of control tissue with tissue treated with a particular drug to study the effects of a potential medical treatment. To do this, the normal state and the diseased state are compared before and after administration of the medication.

The microarray technique holds significant importance in the field of animal genetics for several reasons:

Genetic Variation Analysis: Microarrays allow researchers to analyse genetic variations within animal populations. By studying thousands of genetic markers simultaneously, microarrays facilitate the identification of genetic variants associated with desirable traits, such as disease resistance, productivity, or adaptation to specific environments.

Gene Expression Profiling: Microarrays enable the comprehensive study of gene expression patterns in animals. By examining the expression levels of thousands of genes simultaneously, researchers can gain insights into the molecular mechanisms underlying various biological processes, such as development, immune response, metabolism, and behavior.

Disease Diagnosis and Biomarker Discovery: Microarrays are valuable tools for diagnosing genetic diseases in animals and identifying potential biomarkers for disease detection and monitoring. By comparing gene expression profiles between healthy and diseased animals, researchers can identify molecular signatures associated with specific diseases, leading to the development of diagnostic tests and targeted therapies.

Breeding and Selection Programs: Microarrays can aid in animal breeding and selection programs by facilitating the identification of genetic markers linked to economically important traits, such as milk production in dairy cattle, meat quality in livestock, or disease resistance in poultry. This information can be used to inform breeding decisions and accelerate genetic improvement efforts.

Pharmacogenomics and Toxicogenomics: Microarrays play a crucial role in pharmacogenomic and toxicogenomic studies in animals. By analysing gene expression changes in response to drugs or environmental toxins, researchers can elucidate drug mechanisms of action, predict drug responses, and assess the safety of pharmaceuticals and chemicals in animals (Davidescu et al., 2020; Davidescu et al., 2021; Davidescu et al., 2022).

Overall, the microarray technique is a powerful tool for advancing our understanding of animal genetics, improving animal health and welfare, and enhancing breeding and selection programs in livestock, poultry, aquaculture, and companion animals.

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RESEARCH ON CURRENT TRENDS IN BREEDING KARAKUL OF BOTOSANI SHEEP - BLACK AND GREYISH LINE

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Abstract

Taking into account the decreasing trend in skin production, Karakul of Botoșani sheep breeders have reoriented towards meat production, as the demand for this production is increasing, both in the country and for export, Karakul of Botosani sheep meat being highly appreciated in Arab countries. We want to capture the effect of increased meat production on the quality of the curl, which influences the commercial value of the skin. We will analyze the productive capacity of the Karakul of Botosani breed for both skin production and meat production, and the relationship between them, knowing that when you want to improve one production, you do it at the expense of another, for example improved milk production leads to a lower meat production, or a higher milk production leads to a decrease in milk fat percentage, if we are talking about major changes in the productions, because small changes, or changes up to a certain threshold, called limits of productions, do not have major effects on the other productions.

***Key words**: body weight, Karakul of Botosani, pelts, production sheep.*

INTRODUCTION

Karakul of Botosani breed, is well known for its skin production, production that is influenced by the proper quality of the curl and surface characteristics, or the size of the skin. In this study we will analyze the correlation between the weight at lambing and the specific score from appreciation sheet, respectively the influence of the weight at lambing over the skin quality. The larger the skin surface, the better the degree of coverage will be, i.e. less skins will be used to obtain the finished product (jackets, gloves, vests, hats, etc.).

A study was conducted to investigate whether simple measurements taken on the carcass post-mortem could be used to accurately predict composition and key meat quality traits. (Lamber et al., 2009).

The skin surface is in close correlation with the birth body weight, so as the weight increases, the skin surface is becoming larger.

Body development of the lamb and skin reserves in the flank and neck regions influence the size of the skin surface. When we refer to the surface of the skin, we must consider two reasons, the total surface and the useful surface, since the useful surface has an important role in establishing the commercial value of the skin (Pascal, 2011; 2015; Pascal et al., 1994).

Recent research shows that lambs from the Black and Greyish lines have higher average birth weights compared to lambs belonging to the other lines within the Karakul of Botosani breed (Crîșmaru et al., 2022; Pascal & Nechifor, 2014).

The useful surface is the portion of the total surface covered with curls but excluding the axillary regions, the limbs from the knees and hock and possibly other portions covered with slick hair or wadding. In Karakul of Botosani lambs, the useful surface reaches up to 95% of the total surface (Pascal, 1994).

Climate conditions pay a defining role in sheep production (Sejian et al., 2017; Pascal et al., 2023).

Differences between breeds in the response variable measured were not influenced by diet, sex, or location (Crouse et al., 1981).

A slight influence over the late development of the lambs has the age that they are introduced

at breeding (Florea et al., 2020; Nechifor et al., 2022).
Considering that the curling (i.e. the useful surface) is of the same quality, the skin value is the higher the larger its surface is. The quality of the curl is given by the score value obtained in the appreciation sheet.

MATERIALS AND METHODS

The biological material analyzed was represented by a number of 1378 Karakul of Botosani individuals from Black and Greyish color lines. The entire herd subject to evaluation is included in the Genealogical Register of the breed and is in the character improvement program for skin quality.
The study was carried out during five consecutive years, the lambs being assessed at birth according to the assessment norms of Karakul of Botosani sheep subject to official production control.
The assessment of body weight was carried out with the help of the electronic scale, 24-48 hours after lambing, in the same time with the appreciation sheet.
For the objective assessment of the skin surface, we will focus on the body weight at birth, since the higher the weight of a lamb at birth, the larger the skin surface is and a better later body development.
At birth, each individual, had an appreciation sheet filled in and was weighed, obtained values were grouped according to the color line and according to the individual's destination. The data thus obtained were systematized and processed statistically. 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 variant and the standard deviation of the observed character. Statistics are written with Latin letters: arithmetic mean ($\overline{X}$), variation (s^2), standard deviation (s).
The collected data were processed using the spreadsheet application MsExcel 2013.

RESULTS AND DISCUSSIONS

Depending on the destination of the evaluated products, the data were statistically processed for lambs kept for reproduction, for those destined for meat and for those for skin production.
According to the data presented in Table 1 both in the Greyish and Black line at the individuals kept for reproduction, the skin quality improved during the analyzed period, thus in the greyish individuals, in 2019, we have individuals with an average score of 513.64 points and an average lambing weight of 3.69 kg and in 2023 the score increases to an average of 529.40 points and an average lambing weight of 5.53 kg. The same aspect can be observed in individuals from the Black line, respectively in 2019 we record an average score of 521.98 points and an average lambing weight of 3.74 kg and in 2023 we obtain an average score of 527.35 points and an average lambing weight of 5.55 kg. In greyish individuals, the highest average value of the score was recorded in 2023, respectively 529.40 points, and the highest value of the average weight at lambing was recorded in 2021, respectively 5.56 kg, a weight close to that one recorded in 2023. At black individuals, the highest average value of the score was recorded in 2023, respectively 527.35 points, and the highest value of the average weight at lambing was recorded in 2021, respectively 5.75 kg, a weight close to that recorded in 2023. Comparing the year 2019 and 2023 the average score, respectively the curl quality, did not show significant differences, instead the average weight at lambing increased by 1.84 kg in the case of greyish individuals and 1.81 kg in the case of black individuals. As the average lambing weight and the curl quality have been improved, it only increases the commercial value of the skins, ie skins of a better quality and with a larger usable area.
The best results were found in 2020, in terms of curl quality with an average score of 516.65 for individuals from the Greyish line and 534.17 points for individuals from the Black line and in terms of average weight at lambing the best values were obtained in 2021, respectively 5.56 kg for individuals from the Greyish line and 5.75 kg for individuals from the Black line
The category of individuals destined for breeding plays a decisive role in the improvement of the breed, as they represent the future generations of parents, and by keeping

the most valuable individuals at the breeding ground, we ensure that in the future we will obtain homogeneous and quality individuals, to achieve a economic efficiency of the unit as high as possible. A not to be neglected production of the Karakul of Botosani breed is also that of meat, this because recently the buyers interest in skins has decreased, and this has led the Karakul of Botosani sheep breeders to reorient their production towards that of meat, lambs being highly valued in Arab countries.

Table 1. Evaluation of Karakul of Botosani individuals - Greyish line and Black line for reproduction

Year	N (reproduction individuals)	Greyish line									
		Sheet appreciation score					Lambing weight (kg)				
		$\overline{X}$	s	s^2	Max.	Min.	$\overline{X}$	S	s^2	Max.	Min.
2019	14	**513.64**	41.275	1,704	590	455	**3.69**	0.72	0.52	5.00	2.60
2020	20	**516.65**	42.751	1,828	610	455	**4.94**	0.893	0.8	7.10	3.70
2021	39	**503.05**	38.472	1,480	640	415	**5.56**	0.81	0.66	7.00	4.00
2022	24	**509.71**	38.276	1,465	605	430	**4.86**	0.89	0.79	6.20	2.80
2023	35	**529.40**	36.631	1,342	610	465	**5.53**	0.746	0.56	6.90	4.00
Year	N (reproduction individuals)	Black line									
		Sheet appreciation score					Lambing weight (kg)				
		$\overline{X}$	s	s^2	Max.	Min.	$\overline{X}$	s	s^2	Max.	Min.
2019	112	**521.98**	40.382	1,631	610	423	**3.74**	0.448	0.2	5.00	3.00
2020	116	**534.17**	54.467	2,967	650	413	**5.48**	0.946	0.89	9.20	3.50
2021	90	**511.77**	40.732	1,659	613	445	**5.75**	1.02	1.05	7.60	3.40
2022	107	**501.00**	41.489	1,721	635	400	**5.23**	0.945	0.89	9.50	3.10
2023	83	**527.35**	34.382	1,182	620	465	**5.55**	0.899	0.81	7.90	3.30

Table 2. Evaluation of Karakul of Botosani individuals - Greyish line and Black line for meat production

Year	N (individuals for meat)	Greyish line									
		Sheet appreciation score					Lambing weight (kg)				
		$\overline{X}$	S	s^2	Max.	Min.	$\overline{X}$	s	s^2	Max.	Min.
2019	12	**471.58**	36.75	1,351	525	408	**3.46**	0.431	0.19	4.40	3.00
2020	14	**452.42**	37.916	1,438	515	375	**5.28**	0.72	0.52	6.00	3.70
2021	25	**514.16**	57.369	3,291	598	415	**4.67**	0.9	0.81	6.80	3.50
2022	13	**468.77**	41.944	1,759	535	400	**4.48**	0.997	0.99	6.10	2.30
2023	6	**518.33**	47.293	2,237	590	475	**4.77**	0.877	0.77	5.70	3.30
Year	N (individuals for meat)	Black line									
		Sheet appreciation score					Lambing weight (kg)				
		$\overline{X}$	s	s^2	Max.	Min.	$\overline{X}$	s	s^2	Max.	Min.
2019	54	**494.48**	40.224	1,618	595	423	**3.50**	0.349	0.12	4.20	2.40
2020	32	**481.09**	40.36	1,629	575	385	**5.37**	0.988	0.98	6.80	3.00
2021	143	**499.43**	40.792	1,664	580	405	**4.94**	0.92	0.84	7.20	3.00
2022	149	**469.21**	28.885	834	555	310	**4.63**	0.832	0.69	7.00	2.90
2023	93	**495.70**	32.87	1,080	575	420	**4.70**	0.784	0.61	6.40	2.80

In lambs destined for meat, the quality of curling is not a primary objective, this can also be seen in the centralized data in Table 2 where the average score for greyish individuals, in 2019, was 471.58 points, but still improved in 2023 to 518.33 points and for black individuals the average score had similar values in the analyzed period, with 494.48 points in 2019 and 495.70 points in the year 2023. For meat production, the average weight at lambing is important, which influences the further body development of the lamb, so if in 2019 the average weight at lambing of greyish individuals was only 3.46 kg, in 2023 it reached 4.77 kg, recording a difference of 1.31 kg and in 2020 was recorded the highest average lambing weight, respectively 5.28 kg. In black individuals in 2019 the average lambing weight was only 3.50 kg, in 2023 it reached 4.70 kg, registering a difference of 1.20 kg and in 2020 the highest average lambing weight was recorded, respectively 5.37 kg. By improving the average lambing weight, in addition to the economic efficiency related to meat production, an improvement in the useful area of the skin was ensured if these individuals were directed to the skin production.

Table 3. Evaluation of Karakul of Botosani individuals - Greyish line and Black line for skin production

Year	N (individuals for skin production)	Greyish line									
		Sheet appreciation score					Lambing weight (kg)				
		$\overline{X}$	s	s^2	Max.	Min.	$\overline{X}$	s	s^2	Max.	Min.
2019	2	**510.00**	7.071	50	515	505	**4.00**	0	0	4.00	4.00
2020	2	**460.00**	7.071	50	465	455	**3.85**	0.07	0.005	3.90	3.80
2021	9	**490.11**	16.929	287	528	470	**4.21**	0.4	0.16	5.00	3.70
2022	5	**476.00**	47.88	2,293	535	430	**3.24**	0.23	0.05	3.60	3.00
2023	2	**477.50**	53.033	2,813	515	440	**3.95**	0.636	0.41	4.40	3.50
Year	N (individuals for skin production)	Black line									
		Sheet appreciation score					Lambing weight (kg)				
		$\overline{X}$	s	s^2	Max.	Min.	$\overline{X}$	s	s^2	Max.	Min.
2019	22	**488.55**	35.981	1,295	568	425	**3.06**	0.412	0.17	3.80	2.20
2020	18	**502.35**	37.993	1,443	578	440	**5.08**	1.325	1.76	7.80	2.50
2021	62	**500.19**	30.469	928	575	375	**4.31**	0.52	0.27	6.40	3.00
2022	51	**465.75**	37.083	1,375	610	340	**3.47**	0.785	0.62	5.60	2.10
2023	24	**489.58**	36.083	1,301	525	400	**4.05**	1.25	1.56	6.20	2.30

During the analyzed period, the individuals for skins production came from twin lambings or isolated cases when the lambs had a low birth weight and their mothers do not have enough milk to ensure their latter development. According to the centralized data in Table 3 we can see that in the greyish individuals the skin quality, for the slaughtered products, decreased comparing the year 2019 with 510.00 points to the year 2023 when 477.50 points, but the average weight at lambing does not register significant differences, being 4.00 kg in 2019 and 3.95 kg in 2023. In black individuals, the skin quality did not register significant differences, respectively 488.55 points in 2019 and 489.58 points in 2023, but regarding the average weight at lambing it recorded 3.06 kg in 2019 and 4.05 kg in 2023, with a difference of 1.01 kg and with the highest average weight recorded in 2020, respectively 5.08 kg. If in the greyish individuals the quality of the curl and the useful surface did not undergo major changes, also due to the small number of products sacrificed for the skins, in the black individuals the quality of the curl remained almost unchanged, but the useful surface of the skin increased.

The overall picture regarding the quality of the curl and the average lambing weight of individuals from the Black and Greyish lines, during the analyzed period, is shown in Table 4, Figure 1 and Figure 2.

As we can see in Table 4 in individuals from the Greyish Line, both the curl quality (expressed by the average score from the appreciation sheet) and the average lambing weight improved during the analyzed period, so in 2019 we have an average score of 495.36 points and an average weight of lambing of only 3.61 kg and in 2023 we have an average score of 525.44 points and an average lambing weight of 5.35 kg. In individuals from the Black Line, the quality of the curl did not register significant differences, in 2019 we have 510.17 points and in 2023 we have 508.10 points, instead the average weight at lambing was improved from 3.59 kg in 2019 to 4.97 kg in 2023.

Due to the current trends in the skins market, i.e. a decrease in their demand, Karakul of Botosani sheep breeders tend to reorient themselves towards meat production, this aspect is primarily observed through the prism of the fact that the average weight at calving had a spectacular jump, i.e. 1.74 kg for individuals from the Greyish line and 1.38 kg for individuals from the Black line. The increase in the average weight at lambing was achieved by the use of male specimens with greater body development. Although the average weight at lambing was much higher in 2023, the quality of the curl did not suffer, on the contrary, in the products from the Greyish line, it even improved, reaching 525.44 points, and in the products from the Black line, they had a value close to the one recorded in 2019.

Contrary to expectations, as it is known that when you improve one production you do it at the expense of another, for example if you improve milk production we will have a lower meat production, in the Karakul of Botosani breed it can be observed that it is still possible to go in parallel with the improvement of two productions, that of meat and that of skins, but the leap from the skin quality does not compare with the leap gained in body development, that is for meat production.

Table 4. Evaluation of Karakul of Botosani individuals - Greyish line and Black line overall

Year	N (total individuals)	Greyish line									
		Sheet appreciation score					Lambing weight (kg)				
		$\overline{X}$	s	s^2	Max.	Min.	$\overline{X}$	s	s^2	Max.	Min.
2019	28	**495.36**	42.577	1,813	590	408	**3.61**	0.592	0.35	5.00	2.60
2020	36	**488.53**	50.47	2,547	610	375	**5.01**	0.857	0.73	7.10	3.70
2021	73	**505.26**	44.372	1,969	640	415	**5.09**	0.95	0.91	7.00	3.50
2022	42	**493.02**	44.105	1,945	605	400	**4.55**	1.004	1.008	6.20	2.30
2023	43	**525.44**	39.36	1,549	610	440	**5.35**	0.848	0.72	6.90	3.30
Year	N (total individuals)	Black line									
		Sheet appreciation score					Lambing weight (kg)				
		$\overline{X}$	s	s^2	Max.	Min.	$\overline{X}$	s	s^2	Max.	Min.
2019	188	**510.17**	42.209	1,782	610	423	**3.59**	0.471	0.22	5.00	2.20
2020	166	**519.57**	56.174	3,155	650	350	**5.42**	0.997	0.99	9.20	2.50
2021	295	**503.36**	39.112	1,530	613	375	**5.05**	1.03	1.05	7.60	3.00
2022	307	**479.72**	38.329	1,469	635	310	**4.65**	1.046	1.09	9.50	2.10
2023	200	**508.10**	37.481	1,405	620	400	**4.97**	1.036	1.07	7.90	2.30

As seen in Figure 1 the average score recorded a tortuous evolution, with relatively close values, the only exception is found in individuals from the Greyish Line who were destined for meat and in which the average score recorded the most different values, respectively with maxima in the years 2021 and 2023 and with lows in 2019, 2020 and 2022.

As seen in Figure 2 the average weight at lambing was improved during the analyzed period, the lowest values were recorded in 2019 with only one exception, respectively in the individuals sacrificed for skins at the Greyish line, where the lowest value was obtained in 2022, respectively 3.24 kg, this fact being due to the very small number of products slaughtered for skins that year.

The influence of rams used in breeding to improve meat production can be seen through the fact that since 2020, both the reproduction individuals and the meat individuals have increased the average birth weight by at least 1 kg.

This fact proves to us that the Karakul of Botosani breed can always manage the trends in the market, i.e. if at the moment meat production is required, then it can be directed towards improving this production, and if the market trend returns to the skin production, surely the Karakul of Botosani sheep can return to improve the quality of the curls and the useful surface, because during the analyzed period this aspect did not suffer a regression but on the contrary had, although insignificant, an improvement.

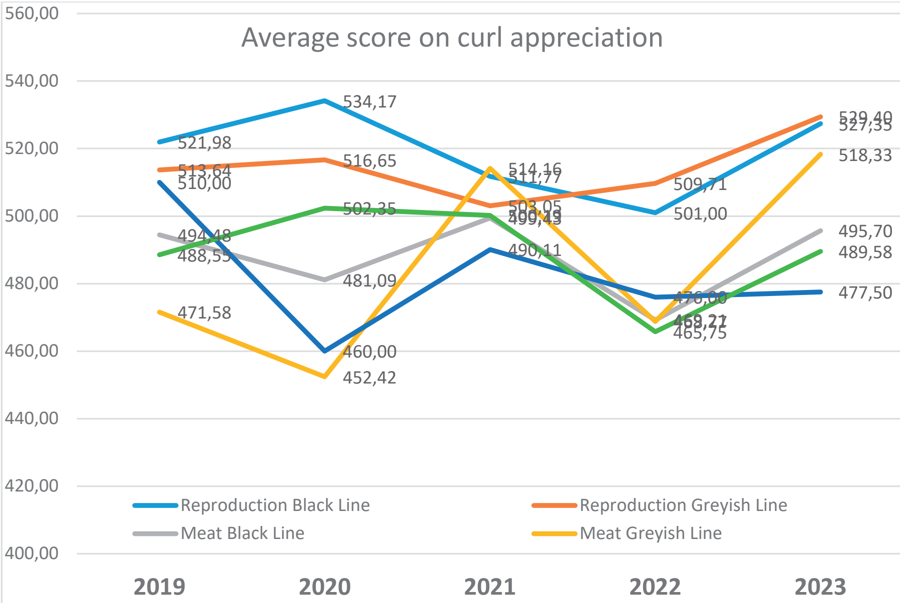

Figure 1. Dynamics of average score for curl appreciation at Black and Greyish lines during the analyzed period

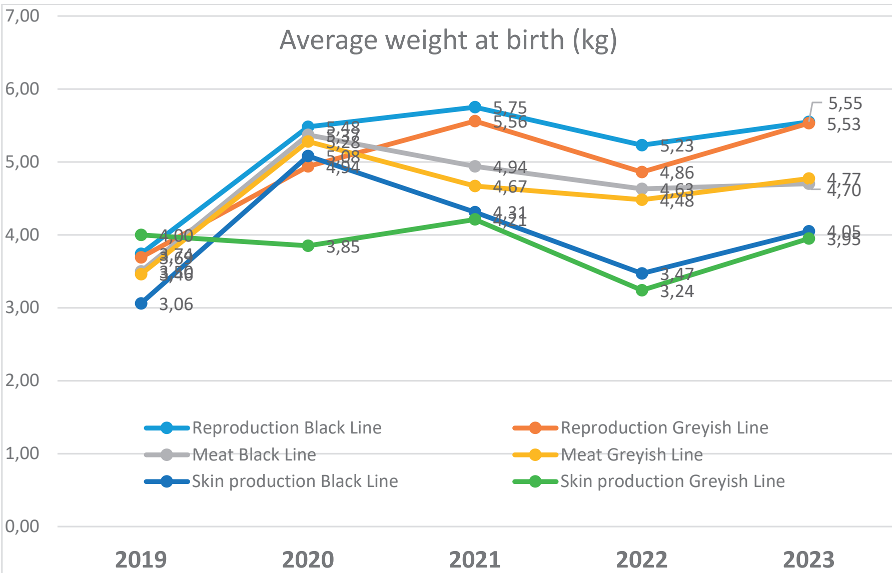

Figure 2. Dynamics of lambs average birth weight for Black and Greyish lines during the analyzed period

CONCLUSIONS

The interest in the skin production is decreasing, so the Karakul of Botosani sheep breeders are oriented towards increasing meat production, by obtaining products that have, from birth, greater weights, a fact that will help them in their latter growth and development.

For the massiveization of individuals from the Black and Greyish Lines, breeders (rams) with a body weight as high as possible are used, and this fact is observed with the assessment of the average weight at lambing, which reaches a maximum of 7.10 kg in 2020, at Greyish lambs and respectively 9.50 kg in 2022 for lambs from the Black line.

Every year, the lambs with a body weight at lambing as high as possible, and a high curl quality were kept for reproduction, in order to have in the following generations valuable parents for both meat and skin production.

The category of lambs slaughtered for skin production was represented by lambs from twin lambs or underdeveloped lambs whose mothers did not have enough milk to ensure their correct development.

The highest average weight at lambing was obtained for the Black variety in 2020, respecttively 5.42 kg, and the best average skin quality score was obtained in 2023, for lambs from the Greyish line, respectively 525.44 points.

The Karakul of Botosani breed can always manage the trends in the market, i.e. if at the moment meat production is required, then it can be directed towards improving this production, and if the market trend returns to skin production, with certainty the Karakul sheep of Botosani can return to improve the quality of the curls and the useful surface.

The fact that the individuals with the best score in terms of the quality of skins can be found in those kept for reproduction, shows us the desire of the Karakul of Botosani sheep breeders to continue the activity of raising sheep for skin production in the hope that the demand for this production will return.

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MILK PRODUCTIVITY AND BREEDING-GENETIC PARAMETERS OF ECONOMICALLY VALUABLE TRAITS OF HOLSTEIN COWS OF FRENCH SELECTION

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Abstract

The article presents the results on the study of key economically beneficial traits of pedigree French Holstein cows in the herd of Society of limited liability "Holstein." It was found that the milk yield of cows in the first lactation averaged 8119 kg of milk, and in the second lactation, it was 8226 kg of milk, which is by 107 kg more than in the first lactation. Comparative analysis of the milk productivity of locally generated cows and their mothers revealed that the daughters' yields (cows of local generation) during the first lactation period of 305 days exceeded the mothers' yields by 1367 kg of milk. In the second lactation, the average yield of locally generated cows was 8505 kg of milk, which is by1253 kg more than the average in the first lactation. A positive correlation was established for milk yield during the 305-day lactation period between the first and second lactations (+0.201). The heritability coefficient for lactation yield was determined to be (h^2 = 0.402).

***Key words**: correlation, heritability coefficient, Holstein breed of French selection, lactation, local generation.*

INTRODUCTION

The Holstein breed has become a leading breed worldwide primarily due to strict and purposeful breeding efforts. It is recognized as a global leader in milk productivity and a set of qualities that allow for better adaptation to the conditions of highly mechanized farms and progressive livestock management technologies (Nogaeva, 2019). The Holstein breed is widely used in many countries with diverse climatic conditions, both for improving local dairy breeds and for purebred breeding (Stolcman, 1980; Shendakov, 2005; Altergot, 2013; Gridin et al., 2013; Ivanova, 2019).

The significance of the Holstein breed, bred in the USA, Canada, and several European countries, has recently increased significantly, including in the Republic of Moldova. Cow's milk and products made from it constitute a substantial part of the population's diet in these countries (Lebedko et al., 2020).

The breeding work plays a crucial role in increasing milk production, as it determines the genetic progress of breeds and herds (Gridin et al., 2019; Ivanova et al., 2021). All other factors, including technological elements, only contribute to the realization of this process.

For successful breeding work in a herd of cattle, it is important to know the phenotypic variability of animal productivity, heritability, and the correlation coefficient between economically valuable selection traits and other parameters (Litovchenko, 2007; Ignatyeva & Lavrentiev, 2020). Variability is influenced by differences in the genotype of animals and external environmental factors that have varying effects on the development of a trait in different individuals. Therefore, the degree of variability in milk yield and other traits is not the same in different herds and populations of cattle. According to literature data, milk fat and protein content, as well as the live weight of cows, are considered more stable indicators, meaning they are less variable (Tamarova, 2005; Seltsov et al., 2012).

Among the key selection traits for cattle are milk yield and milk fat content.

It is known that animals, despite having approximately equal heritability, exhibit different trait formations under the influence of various environmental conditions. The

phenotypic diversity of animal traits is determined by the complex interaction of genetics and living conditions (Baumgard et al., 2011; Berman, 2011; Gorlov et al., 2016).
The magnitude of these traits is influenced by breeding methods, the achieved level of herd cow productivity, feeding and housing conditions, and other factors. Therefore, population-genetic parameters of identical traits in different herds can vary significantly.
The study of milk productivity was conducted on Holstein cows of French breeding (Holstein crossbreeds) in the herd of SLL "Holstein". It is noteworthy that France ranks second in the European Union in terms of whole milk production, accounting for 22.1% of the total production (Medvedeva & Penkov, 2013). Among the 12 major dairy cattle breeds, five have high demand for export: Holstein crossbreeds, Montbeliarde, Normande, Abondance, and. Among them, Holstein crossbreeds exhibit the highest productivity, with an average yield of 9135 kg of milk, protein content of 3.30% over a lactation period of 305 days (Kuba, 2017).
The aim of the research is to study the variability, correlation, and heritability of milk productivity traits at Holstein cows of French breed in the SLL "Holstein" herd.

MATERIALS AND METHODS

The research focused on the population of Holstein cows of French selection in the breeding herd of the Society of Limited Liability (SLL) "Holstein" located in Roshkan village, Anenii Noi district, in the amount of 308 heads. The breakdown by lactations included the first (n = 142), second (n = 72), third (n = 24), fourth (n = 27), fifth (n = 27), and sixth (n = 14) lactations.
The study of the productivity of Holstein cows of French selection involved the use and analysis of pedigree certificates for heifers, pedigree cards for cows, records of milk productivity, and other documents from primary zootechnical records. Evaluation and analysis of cows based on milk productivity were conducted using commonly accepted methods, considering yield over 305 days of lactation, fat content in milk, milk fat production for the lactation, and assessing the variability of these indicators (Cv).
The genetic potential of first calf's productivity was determined using the Parental Index of Cows (PIC). The Parental Index was calculated using the Wright path coefficient (Krasota & Dzhaparidze, 1999).
To study the variability and heritability of milk productivity traits in the first lactation, mother-daughter pairs were selected. Each sample consisted of 105 cows and 105 offspring. Parameters studied included yield over 305 days of lactation, fat content in milk, milk fat quantity, and the variability of these indicators (Cv).
The calculation of selection-genetic parameters of productivity and the statistical processing of research materials were conducted using the methodologies proposed by Plokhinsky (1970) and Merkuryeva (1983). The coefficient of correlation (r) was computed on a computer using the CORREL function within the Excel software environment. The heritability of selection traits was determined using the method of doubled correlation ($h^2 = 2r$). The numerical data the research were biometrically processed on a personal computer using Microsoft Excel 2010 software.

RESULTS AND DISCUSSIONS

As a result of studying the main economically valuable traits of Holstein cows of French selection in the SLL "Holstein" herd, it was determined that the average milk yield of the studied population of cows in the first lactation was 8119 kg of milk, as shown in Table 1.
Starting from the third lactation, a decrease in milk yields is observed. In comparison to the first lactation, yields decreased by 329 kg (third lactation), 378 kg (fourth lactation), 440 kg (fifth lactation), and 931 kg (sixth lactation) of milk, with $P<0.01$, $P<0.005$ and $P<0.001$ significance levels, respectively.
As is known, the degree of genetic diversity in a herd is judged by the variability of economically valuable traits. The longer the breeding work was conducted and the more intense was the selection, the smaller would be the magnitude of variability in economically valuable traits. The results of the analysis of selection-genetic parameters of economically valuable traits in the SLL "Holstein" herd, depending on lactation, are presented in Table 2.

Table 1. Characteristics of cows by milk productivity for a range of lactation (X ± Sx)

Lactation	Number of cows, n	Indices			
		Milk yield, kg	Fat content, %	Amount of fat, kg	Body weight, kg
The first	142	8119±52.8	3.83±0.003	312±2.1	639±2.8
The second	72	8226±71.2	3.84±0.004	313±3.8	681±4.7
The third	24	7790±96.9 **	3.84±0.01	299±3.7	687±1.8
The fourth	27	7741±85.6 *	3.83±0.01	296±3.2	695±3.6
The fifth	29	7679±97.3***	3.83±0.01	294±3.5	692±3.4
The sixth	14	7188±95.3***	3.82±0.01	274±3.4	695±3.3
Average	308	8001±36.0	3.83±0.002	307±1.5	663±2.7

Table 2. Coefficient of variation for milk productivity indicators (Cv, %)

Indices	Lactation					
	I	II	III	IV	V	VI
Milk yield	7.8	3.0	1.8	5.8	6.8	5.0
Fat content	0.9	0.9	0.9	0.7	0.7	1.2
Amount of fat	7.9	10.3	6.1	5.6	6.4	4.7
Live weight	2.8	3.0	1.1	1.5	1.7	1.6

It should be noted that the coefficients of variation for all studied productivity traits were below the normative data. However, the lowest indicators were identified for milk fat content. In the research results (Bashchenko et al., 2020; Kruglyak & Biryukova, 2007), it is also noted that the degree of variation in milk fat content is unusually low and amounts to 1.3%.

Low values of coefficients of variation indicate a reduction in genetic diversity and homogeneity in the herd of "Holstein" cows at SLL. Similar data were obtained in research results in 2022 by (Konstandoglo et al., 2023).

To determine the indirect effect of selection on correlated traits, correlation coefficients are used. Differences in this population parameter were identified across lactations, as shown in Table 3. The correlation between milk yield and the percentage of fat in the milk of cows in the first-fifth lactations showed a negative correlation.

Table 3. Genetic correlation between milk productivity indicators in lactation dynamics, $r \pm m_r$

Indicators	First lactation	Second lactation	Third lactation	Fourth lactation	Fifth lactation
Milk yield - Fat content, %	-0.397±0.07	-0.617±0.06*	-0.696±0.05***	-0.489±0.08	-0.438±0.01
Milk yield - Amount of fat, kg	0.972±0.02	0.067±0.08	0.990±0.002	0.982±0.04	0.987±0.00

Note: * - P < 005; *** - P < 0.001

A comparative analysis of the correlation results between yield and the percentage of fat revealed 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 evident, the unidirectional selection based on the yield level led to an increase in the negative correlation between these traits, complicating the conduct of successful selection and emphasizing the need for simultaneous selection based on both yield and fat content in the milk.

In the SLL "Holstein" herd, the milk productivity of locally bred cows and their mothers was studied for the first 305 days of lactation, Table 4.

Table 4. Comparative characteristics of locally bred Holstein cows and their mothers based on milk productivity for the first lactation

Indices	Number of cows, N	Milk yield for 305 days, kg		Fat content, %		Amount of fat, kg	
		X ± Sx	Cv, %	X ± Sx	Cv, %	X ± Sx	Cv, %
Daughters	105	7716±63.5***	8.4	3.84±0.005	1.2	297±2.3***	8.0
Mothers	105	6349±41.9	6.8	3.97±0.005	1.3	252±1.6	6.4

Note: ** - P < 0.01; *** - P < 0.001

Through a comparative analysis of the milk productivity of locally bred cows and their mothers, it was found that the yields of daughters (locally bred cows) exceeded the yields of their mothers by 1367 kg of milk. In terms of milk fat quantity, daughters also had an advantage of 45 kg.

Accordingly, the coefficients of variation for yield and milk fat quantity in locally bred cows were slightly higher than the analyzed traits in mothers. However, it is worth noting that the range of variability is not wide for the analyzed population of cows, leading to a reduction in their genetic diversity and an increase in the level of homozygosity.

It is important to highlight that 54 locally bred cows completed their second lactation. The results of the study of productivity for the first two lactations of locally bred cows, as well as correlation and heritability indicators, are presented in Table 5.

Table 5. Milk productivity of locally bred cows, correlation between lactations (r), and heritability (h^2), X ± Sx

Lactation	Number of cows, n	Milk yield		Fat content, %	Cv,%	Amount of fat, kg	Cv, %
		kg	Cv, %				
First	54	7252±78.1	7,5	3.85±0.01	1.1	296±12.1	8.4
Second	54	8505±50.0***	4,3	3.84±0.003	0.6	323±4.3	9.8
Correlation (I-II), r		0.201±0.13		-0.152±0.14		0.037±0.14	
Heritability, h^2		0.402		0.304		0.074	

The comparative analysis productivity in locally bred cows showed that during the second lactation, the average milk yield was 8505 kg, which is by1253 kg higher than the average for the first lactation. The variability of milk yield and fat content for the second lactation is slightly lower than for the first lactation. However, for this trait, it is a relatively low indicator, as it is known that in most herds, the coefficient of variability for milk yield is at least 20-25%, and for fat content, it is at least 5-7% (Seltsov et al., 2012).

A positive correlation for milk yield over 305 days of lactation was found between the first and second lactations (0.201), with a weak relationship.

The heritability coefficient for milk yield over lactation was (h^2 = 0.402). This allows us to conclude that heritability for milk yield was predominantly influenced by genetic factors.

SLL "Holstein" continues to import breeding animals, and in 2022 - 32 heifers were imported. The study of milk productivity indicators for first-calf heifers at the end of lactation showed that the milk yield averaged 8734 ± 57.6 kg, fat content was 3.82 ± 0.003%, and the amount of milk fat averaged 335 ± 2.5 kg.

The study of milk production indicators at imported pregnant heifer ancestors (Table 6) demonstrated that the average milk production of mothers was 8378 kg, fat content was 3.93%, and the total fat was 331 kg.

Table 6. Milk productivity index at ancestors, French-origin Holstein breed (X ± Sx)

Indices	Mother	Cv, %	Mother's Mother	Cv, %	Father's Mother	Cv, %
Herd size, n	26		26		25	
Milk, kg	8378±354.2	21.6	8946±361.1	20.6	10851±432.1	19.9
Fat content, %	3.93±0.09	11.3	4.02±0.08	10.2	4.08±0.07	8.5
Fat quantity, kg	331±14.1	21.7	357±12.5	17.9	438±17.7	19.8

The average milk production of maternal grandmothers was 8946 kg, with a fat percentage of 4.02, and the total fat amount was 357 kg. The analysis revealed that the milk yield of maternal grandfathers in the highest lactation was 10851 kg.

The coefficients of variability (Cv) for milk yield in the analyzed groups of mothers ranged from 19.9% (mothers of fathers) to 21.6% (mothers). Regarding fat content and the quantity of milk fat, it is noteworthy that the values of these traits in the sample on average also corresponded to literature data.

To assess the potential capabilities of animals for all indicators of female ancestors, there were calculated the Breeding Index of Cows (BIC) and the realization of the genetic potential of first-calf heifers (Table 7).

Table 7. Realizing of the Genetic Potential of dairy productivity

Parental Index of Cows (PIC)		Own productivity		Realization of genetic potential (RGP), %	
Milk Yield, kg (X ± Sx)	Fat Content, % (X ± Sx)	Milk Yield, kg (X ± Sx)	Fat Content, % (X ± Sx)	Milk Yield	Fat Content
9138±375.4	3.99±0.08	8734±57.6	3.82±0.003	95.6	95.7

The analysis of the obtained data revealed that the parental index for milk yield was at the level of 9138 kg and 3.99% for fat content. The realization of the genetic potential for milk yield was 95.6%, and for fat content in milk, it was 95.7%. To assess the heritability indicators, it was used the method of correlating the traits of daughters with their mothers, as shown in Figure 1.

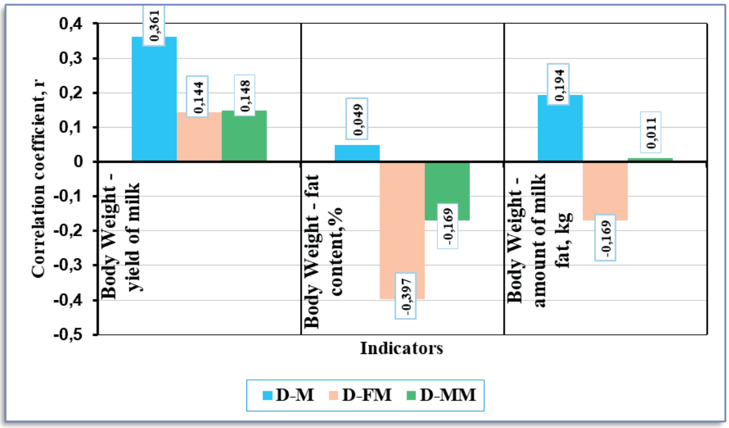

Figure 1. Correlation between Productivity Indicators and Body Weight of Daughters and Maternal Ancestors

The analysis of the milk yield relationship showed that a positive weak connection was established between Daughter-Mother's Mother (D-MM) and Daughter-Father's Mother (D-FM) - 0.148 and 0.144, respectively. A moderate positive correlation was identified between daughters and mothers (D-M) - 0.361.

For fat content in milk, the correlation between daughters and fathers' mothers (D-FM) was negative, with moderate strength (r = -0.397). The main genetic parameter that numerically indicates the proportion of inherited variability of a trait and, therefore, serves as a selection criterion, is the heritability coefficient (h^2). Based on the obtained data, it can be concluded that milk yield and the quantity of milk fat of first-calf heifers were more influenced by heritability (h^2 = 0.29-0.72) and (h^2 = 0.39), respectively, Table 8.

Table 8. Heritability (h^2) of Main Productive Traits

No.	Indices	Daughter - Mother (D-M)	Daughter - Mother's Mother (D-MM)	Daughter - Father's Mother (D-FM)
1.	Milk Yield	0.72	0.30	0.29
2.	Fat Content	0.10	0.09	0.79
3.	Amount of fat	0.39	0.02	0.34

Maternal ancestors had the greatest influence on milk yield and the quantity of milk fat - h^2 = 0.72 (Mother), h^2 = 0.30 ((Mother's Mother), and 0.39 (Mother), respectively. For the fat content in milk, h^2 = 0.1 (M) and h^2 = 0.09 (MM) were more influenced by paratypical factors, as the heritability coefficients for this trait were very low or absent (FM).

CONCLUSIONS

Coefficients of variability for all studied productivity traits were below normative values, indicating a reduction in genetic diversity and homogeneity within the herd of SLL "Holstein." Comparative analysis of Holstein cows of local breeding and their mothers based on milk productivity for the first 305 days of lactation revealed that the milk yield of daughters (local breeding cows) for the first lactation exceeded the milk yield of mothers by 1367 kg, and the difference was significant ($P < 0.001$).

A weak positive correlation was found between milk yield for the first and second lactations (0.201), and the correlation for fat content was weakly negative (-0.152).

The heritability coefficient between the first and second lactations for local breeding cows for milk yield was ($h^2 = 0.402$). Heritability for milk yield was mainly influenced by genetic factors.

A weak positive correlation was found between daughters and mother's mother (D-MM) and daughters and father's mother (D-FM) - 0.148 and 0.144, respectively. A moderate positive correlation was observed between daughters and mothers of daughters - mothers (D-M) - 0.361.

The milk yield and quantity of milk fat at first-calf heifers were primarily influenced by heritability ($h^2 = 0.29$-0.72) and ($h^2 = 0.39$), respectively.

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COMPARATIVE ANALYSIS OF PROCEDURES FOR PREDICTING BREEDING VALUE FOR TRAITS OF ECONOMIC IMPORTANCE IN A SHEEP POPULATION

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Abstract

The objective of the paper is the comparative analysis of three selection methods, from the point of view of breeding values, for production traits (the amount of milk, milk fat and protein) and reproduction traits (prolificacy) in the Palas Milk Line sheep population. Totally, 486 sheep were analyzed. The heritabilities for breeding values were estimated by the REML method, using an animal model for the four traits analyzed, and were: 0.197, 0.209, 0.263 and 0.235. The comparison of the three methods was carried out by means of the Spearman rank correlation, which allows the comparison of the ranking of the same observation units (individuals), on different criteria (different selection methods): The Spearman rank correlation was applied for the following couples of methods: BLUP and BLP; BLUP and own performances and BLP and own performances. The results for each couple were: 0.998, 0.89 and 0.899, for the amount of milk; 0.996, 0.907 and 0.919, for the amount of fat in milk and 0.998, 0.89 and 0.897, for the amount of protein in milk. For prolificacy, the rank correlation values were 0.953, 0.837 and 0.912. These results show that the highest agreement was achieved between the BLUP and BLP methods.

***Key words**: BLP method, BLUP methodology, breeding values, prediction, selection.*

INTRODUCTION

By 2050, global demand for animal-based food products is expected to increase by 70%. Meeting this demand while minimizing environmental impact would necessitate the use of innovative technologies and strategies for improving animal genetic quality (Georges et al., 2019). The selection index is a mechanism for determining an animal's breeding value by collecting all relevant information on the animal and its relatives. It provides the most accurate linear estimate of an individual breeding value. When records are available from various sources, such as records on the animal, its dam, half sibs, progeny, and so on, it is obviously preferable to use all records to estimate the breeding value (Endris, 2020). Knowing the genetic correlations (rg) and heritability (h^2) of the variables included in the breeding aim and selection index is necessary for breeding value estimation (Scholtens, 2016). Selection is an important aspect of animal breeding and is usually done on the basis of a collection of qualities, each of which is distinguished by its breeding value and economic value. As a result, a recalculation of the economic importance of profit-related traits in sheep is required for the adaptation of the enterprises' breeding objectives based on their economic importance (Slavova, 2022). Genetic improvement is a fundamental means of boosting livestock productivity, and to attain genetic benefits, well-designed animal genetic improvement schemes are required (Haileselassie et al., 2022). The accepted method for genetic evaluation is best linear unbiased prediction (BLUP) applied to an animal model. It has the advantage that all available information is optimally considered, and selection or special mating has little to no impact on the judgement. Due to the relatively low number of offspring produced by natural mating in dairy sheep, information from other relatives is even more crucial in this operation

(Grosu & Rotar, 2015). Best Linear Prediction (BLP) is a technique for calculating breeding values based on linear models, it is a fundamental strategy that takes into account individual relationships (descent) and phenotypic information (Mueller et al., 2021). Animals with higher estimated breeding value are expected to produce offspring with more desirable traits because their breeding values indicate their genetic potential to transmit those traits, by selecting animals with higher estimated breeding value as parents, breeders aim to increase the frequency of desired genes in the population, leading to improvements in overall trait expression (Khatib, 2015). The breeding value as a ratio to the population mean provides an assessment of the animal's performance position in relation to the population mean from which it is drawn; the breeding value given in relation to the population mean provides a measure of the animal's genetic potential (Liu et al., 2023). The selection index is a technique for calculating the breeding value of an animal that incorporates all data about the animal and its relatives. It is the most accurate linear prediction for a single improvement value. Using all available records - i.e. information about the animal, mother, half-siblings, offspring, etc. will undoubtedly be the most advantageous approach for estimating the breeding value of the specimen (Endris, 2020). Estimates of heritability can be used to create selection indices, predict genetic response to selection, and assess how much an individual's own phenotype can be depended on for selection. Heritability estimations for multiple economic features are required for effective cattle operations (Lalit et al., 2016). Estimates of a trait's heritability vary between breeds of sheep and alter gradually over time; animal performance data and pedigree data used to detect genetic linkages between those animals are used to evaluate heredity. Heritability is used to calculate genetic evaluations, anticipate response to selection, and assist producers in determining whether it is more effective to increase qualities through management or selection. Heritability also helps explain how genes influence trait expression (Sutera, 2018).

The objective of the paper is the comparative analysis of three selection methods, from the point of view of breeding value, for production and reproduction characters in the Palas Milk Line sheep population. The three selection methods are represented by: selection based on own performance, selection based on the BLP method (Selection Indices) and selection based on the BLUP - Individual Animal Model methodology. The study focuses on the evaluation of two groups of characters in the analyzed herd Milk production, expressed by the amount of milk, milk fat and protein, and reproductive characteristics, especially prolificacy.

MATERIALS AND METHODS

The study focuses on the evaluation of two groups of traits in the analyzed herd Milk production, expressed by the amount of milk, milk fat and protein, and reproductive traits, especially prolificacy. In the study carried out, 486 sheep from the Palas Milk Line were analyzed. The heritabilities used in calculating the breeding values were estimated by the REML method, using an animal model for the four traits analyzed. The three selection methods are represented by: selection based on own performance, selection based on the BLP method (Selection Indices) and selection based on the BLUP - Individual Animal Model methodology. The comparison of the three listed methods was carried out by means of the Spearman rank correlation, which allows the comparison of the ranking of the same observation units (individuals), on different criteria (different selection methods). This correlation study was conducted between the following pairs of methods: (BLUP and BLP), (BLUP and own performances) (BLP and own performances).

Statistical analysis: The data were analyzed with animal model with R software [1, 2]. The model for trait "i" is as follows:

$$P_i = X_i b_i + Z_1 a_i + e_i$$

For two traits, Mixed Model Equations of BLUP can be written as follows:

$$= \begin{bmatrix} X_1' \cdot r^{11} \cdot P_1 + X_1' \cdot r^{12} \cdot P_2 \\ X_2' \cdot r^{21} \cdot P_1 + X_2' \cdot r^{22} \cdot P_2 \\ Z_1' \cdot r^{11} \cdot P_1 + Z_1' \cdot r^{12} \cdot P_2 \\ Z_2' \cdot r^{21} \cdot P_1 + Z_2' \cdot r^{22} \cdot P_2 \end{bmatrix}$$

G and R are variances and covariances matrices for genotypic and environmental effects:

$$G^{-1} = \begin{bmatrix} \sigma_{a1}^2 & \sigma_{a12} \\ \sigma_{a21} & \sigma_{a2}^2 \end{bmatrix}^{-1} = \begin{bmatrix} g^{11} & g^{12} \\ g^{21} & g^{22} \end{bmatrix};$$

$$R^{-1} = \begin{bmatrix} \sigma_{e1}^2 & \sigma_{e12} \\ \sigma_{e21} & \sigma_{e2}^2 \end{bmatrix}^{-1} = \begin{bmatrix} r^{11} & r^{12} \\ r^{21} & r^{22} \end{bmatrix}$$

The elements of G and R were estimated using the formulas:

$$\sigma_{a\,ii}^2 = \frac{\hat{a}_i' \cdot A^{-1} \cdot \hat{a}_i + tr(A^{-1} \cdot C_{ii})}{q}$$

$$\sigma_{a\,jj}^2 = \frac{\hat{a}_j' \cdot A^{-1} \cdot \hat{a}_j + tr(A^{-1} \cdot C_{jj})}{q}$$

$$\sigma_{a_{ij}} = \frac{\hat{a}_i' \cdot A^{-1} \cdot \hat{a}_j + tr(A^{-1} \cdot C_{ij})}{q}$$

$$\sigma_{e\,ii}^2 = \frac{\hat{e}_i' \cdot \hat{e}_i + tr(B_{ii})}{n}$$

$$\sigma_{e_{ij}} = \frac{\hat{e}_i' \cdot \hat{e}_j + tr(B_{ij})}{n}$$

$$\sigma_{e\,jj}^2 = \frac{\hat{e}_j' \cdot \hat{e}_j + tr(B_{jj})}{n}$$

$\sigma_{a_{ij}}$ = additive covariance between trait "i" and "j"

$\sigma_{e\,ii}^2$ = environmental variance for trait "i",

$\sigma_{e_{ij}}$ = environmental covariance between trait "i" and "j"

$$W = \begin{bmatrix} X_1 \oplus X_2 & Z_1 \oplus Z_2 \end{bmatrix};$$

$$B = W \cdot C \cdot W' = \begin{bmatrix} B_{ii} & B_{ij} \\ B_{ji} & B_{jj} \end{bmatrix};$$

$\oplus$ = Direct sum (S.R. Searle, 1982)

$$e_i = P_i - W \cdot \begin{bmatrix} \tilde{b}_1 & \tilde{b}_2 & \hat{a}_1 & \hat{a}_2 \end{bmatrix}$$

For the trait "i", the heritability was obtained as the ratio between the additive genetic variance and the total phenotypic variance (σ_f^2), according to the formula:

$$h_{ii}^2 = \frac{\sigma_{a\,ii}^2}{(\sigma_{a\,ii}^2 + \sigma_{e\,ii}^2)}$$

Genetic correlation according to the formula:

$$rg_{ij} = \frac{\sigma_{aij}}{\sqrt{\sigma^2_{ii} * \sigma^2_{jj}}}$$

Spearman's rank correlation coefficient according to the formula:

$$rs = 1 - \frac{6\Sigma d^2_i}{n(n^2-1)}$$

Where: n - rank number; d - the difference between the rank occupied by the same animal (candidate to selection), in two rankings based on different criteria (different biometric models).

RESULTS AND DISCUSSIONS

Taking into account the all information disponible, the heritabilities and genetic correlations between each pair of traits, have obtained (Table 1):

Table 1. The heritability (on diagonal) and genetic correlations (above the diagonal) between the four traits analized

Traits	Amount of Milk	Milk Fat	Milk Protein	Prolificacy
Amount of Milk	**0.197** ± 0.263	0.836 ± 0.284	0.441 ± 0.757	0.473 ± 0.731
Milk Fat		**0.209** ± 0.278	0.325 ± 0.839	0.451 ± 0.749
Milk Protein			**0.263** ± 0.347	0.149 ± 0.915
Prolificacy				**0.235** ± 0.311

Heritabilities

On the diagonal of Table 1, we can find the heritabilities and their errors (h^2± s.e) for each trait analized (amount of milk, milk fat, milk protein, and prolificacy), used in calculating the breeding values and were the following: 0.197 ± 0.263, 0.209 ± 0.278, 0.263 ± 0.347 and 0.235 ± 0.311, highlighting the fact that these traits have an intermediate genetic determinism. Many studies have been conducted on calculating genetic parameters for four traits, especially heritability. Shihab et al. (2022) found that the heritability rate in Awassi sheep was 0.19, which is a percentage near to what we found in Palas sheep. Another study on local sheep in Iraq estimated heritability for daily milk production was high 0.22 (Raoof & Khidhir, 2023). In Valle del Belice dairy sheep from Italy, heritability estimates for milk production was low 0.15 (Sutera et al., 2021). Genetic-parameter estimation of milk yield in White Maritza sheep, heritability estimates was between 0.35-0.34 (Zhelyazkova et al., 2023). The heritability estimates for milk production traits (milk yield, - fat yield and protein yield) were 0.24, 0.21 and 0.22 respective in dairy sheep (Mucha et al., 2022). The heritabilities of daily milk yield (kg), fat content (%), and protein content (%) were low 0.09, 0.03, and 0.06, respectively, on dairy traits in Istrian sheep (Špehar et al., 2022). In a previous study heritability estimates for milk yield, fat yield and protein yield were low and ranged between 0.060, 0.065 and 0.067 with standard error ranging from 0.037, 0.039 and 0.037 respectively, in Valle del Belice dairy sheep (Sutera, 2018). Genetic parameter estimation to milk yield and fat and protein yield in dairy herds of southern Chile, estimated heritability for milk yield, and fat and protein were 0.16±0.004, 0.44±0.007 and 0.42±0.006, respectively (Uribe Muñoz et al., 2017). Genetic parameter estimates for composite reproductive traits in Baluchi sheep. the heritability for prolificacy was 0.22 (Esmaeili-Fard et al., 2021). The heritability for prolificacy was 0.12 when evaluating genetic parameters in Xinggao sheep (Liu et al., 2023). In another study to estimate heritability in sheep, the percentage was moderate and amounted to 0.23 (Pascal et al., 2019). Estimates of heritability for number of lambs born per ewe lambing was lowly heritable (0.07), and for milk yield, fat yield and protein yield were all moderately heritable (0.32, 0.26 and 0.30) (Murphy, & Thomas, 2016). In another study the heritability for the number of born lambs was low (0.01) (Pelmuş et al., 2019). The heritability estimates for prolificacy traits was low 0.08 traits in Afshari sheep breed (Pourtahmasebian Ahrabi et al., 2021). Scholtens (2016) estimated of heritability values for milk yield, fat yield, protein yield and prolificacy considered in a breeding objective for dairy sheep in New Zealand were 0.25, 0.21, 0.20 and 0.13 respectively. In another study, the estimated heritabilities for milk, fat, protein production, and prolificacy for East Friesian milk sheep were 0.15, 0.09, 0.20, and 0.04 (Hamann et al., 2004).

Genetic correlations

On the off diagonal of table 1, there are the genetic correlations between each pair of the four analized traits, together with their errors (rg ± s.e):

Genetic correlation measures the degree to which the genetic factors influencing one trait also influence another trait, there is a strong positive genetic correlation between the amount of milk and milk fat traits. This means that the genetic factors influencing higher milk production tend to also be associated with higher milk fat content. The genetic correlation between amount of milk and milk protein is positive but weaker compared to amount of milk and milk fat. There is a positive genetic correlation between amount of milk and prolificacy, indicating that some genetic factors associated with higher milk production may also be associated with higher prolificacy (the ability to give birth to multiple offspring at once). The genetic correlation between milk fat and milk protein is positive but relatively weak. There is a positive genetic correlation between milk fat and prolificacy. The genetic correlation between milk protein and prolificacy is positive but quite weak. Genetic correlations between daily milk yield and fat content, daily milk yield and protein content were negative, and fat content and protein content were positive (-0.22, -0.27, and 0.70), respectively on dairy traits in Istrian sheep (Špehar et al., 2022). The genetic association between the amount of milk yield, fat yield, and protein yield was shown to be substantially genetically associated (0.91 to 0.96). Milk fat percentage and protein production were moderately genetically linked (0.61). Milk yield was negatively associated to fat yield and protein yield (-0.31 and -0.34, respectively). Ewe prolificacy was not significantly (> 0.67) genetically linked with milk yield or protein yield, but was slightly negatively (-0.26) correlated with milk fat (Murphy et al., 2017). According to a Eurosheep study, the genetic correlations between milk and fat and protein yields are favorable and relatively strong (between 0.77 and 0.93). Estimates of genetic correlations between milk output and content are negative and highly variable. Correlations between fat and protein yields and their correlations contents vary according on the breed (Eurosheep, 2022). In dairy herds of southern Chile, estimated genetic correlations were -0.285 and -0.331 between milk yield and fat and protein respectively (Uribe Muñoz et al., 2017). Prolificacy was estimated to have low genetic correlations with yield traits (-0.06 to 0.05). Genetic correlation for Milk, fat, and protein yields were all moderately positively (0.91 to 0.96) in dairy sheep (Murphy & Thomas, 2016). Another study significantly positive genetic association between total prolificacy and milk production (0.16) in prolific Chios dairy sheep (Tsartsianidou et al., 2023). For New Zealand dairy sheep, estimated genetic correlations between milk yield, fat yield, protein yield, and prolificacy were taken into account in the breeding objective. Milk yield correlations with fat yield, protein yield, and prolificacy were 0.85, 0.96, and 0.06, respectively; fat yield correlations with prolificacy were 0.76 and -0.03, and protein yield correlations with prolificacy were 0.02 (Scholtens, 2016).

Breeding values

In the Tables 2, 3, 4 and 5 were shown the breeding values for the best 20 animals from sheep population for amount of milk, milk fat, milk protein, and prolificacy, for three selection methods.

Breeding values for amount of fat is presented in Table 3.

Breeding values for amount of protein is evaluated in Table 4.

Breeding values for prolificacy in presented in Table 5.

Table 2. Breeding values for the best 20 animals from sheep population for amount of milk

Animal	BV-BLUP	Rank-BLUP	BV-BLP	Rank-BLP	BV-OP	Rank-OP
453	26.09	1	25.91	1	27.60	1
29	20.75	2	20.66	2	20.66	3
481	19.59	3	19.47	3	19.47	5
253	18.01	4	18.07	4	16.08	8
370	16.66	5	16.38	5	15.78	9
139	16.40	6	16.09	6	15.39	11
335	16.26	7	15.81	7	13.37	21
340	15.30	8	15.09	8	15.57	10
221	15.20	9	15.01	9	11.95	31
452	14.73	10	14.41	11	21.96	2
31	14.50	11	14.27	12	9.64	53
258	14.38	12	14.46	10	13.61	16
99	14.00	13	13.70	14	12.19	30
350	13.89	14	13.78	13	13.58	18
25	13.83	15	13.65	15	20.07	4
445	13.70	16	13.45	16	9.07	57
23	13.46	17	13.20	17	11.53	32
32	13.30	18	13.11	18	7.32	77
321	13.14	19	12.72	20	10.61	43
298	12.90	20	12.45	21	10.78	38

Table 3. Breeding values for the best 20 animals from sheep population for amount of fat

ANIMAL	BV-BLUP	Rank-BLUP	BV-BLP	Rank-BLP	BV-OP	Rank-OP
453	5.07	1	5.00	1	5.80	1
481	4.78	2	4.72	2	4.72	2
29	3.86	3	3.84	3	3.84	4
221	3.62	4	3.51	4	2.95	20
335	3.46	5	3.33	5	3.39	14
340	3.31	6	3.23	7	3.48	8
350	3.29	7	3.30	6	3.08	18
25	3.14	8	3.10	8	4.28	3
211	3.10	9	3.07	9	2.36	36
370	3.07	10	2.86	13	2.86	21

ANIMAL	BV-BLUP	Rank-BLUP	BV-BLP	Rank-BLP	BV-OP	Rank-OP
357	3.05	11	2.96	11	2.56	27
362	2.99	12	3.06	10	3.26	16
446	2.91	13	2.83	14	3.07	19
132	2.80	14	2.90	12	3.40	11
32	2.71	15	2.67	15	2.08	46
81	2.62	16	2.56	18	3.67	6
253	2.53	17	2.56	19	2.47	29
127	2.49	18	2.41	21	3.37	15
369	2.43	19	2.60	16	3.47	9
139	2.36	20	2.28	26	2.37	34

Table 4. Breeding values for the best 20 animals from sheep population for amount of protein

ANIMAL	BV-BLUP	Rank-BLUP	BV-BLP	Rank-BLP	BV-OP	Rank-OP
139	3.05	1	3.03	1	3.19	1
298	3.02	2	2.93	2	3.14	2
321	2.95	3	2.87	3	2.56	6
394	2.62	4	2.68	4	2.58	5
168	2.55	5	2.56	5	2.31	9
482	2.20	6	2.22	6	2.52	7
388	1.98	7	2.05	7	1.73	15
370	1.93	8	1.95	8	2.13	11
25	1.91	9	1.90	9	2.71	4
312	1.87	10	1.79	12	1.12	47
212	1.86	11	1.87	10	1.87	14
423	1.77	12	1.85	11	2.75	3
178	1.72	13	1.74	13	1.72	16
284	1.69	14	1.70	15	1.70	18
481	1.68	15	1.70	16	1.70	19
395	1.67	16	1.73	14	2.19	10
241	1.63	17	1.63	18	1.63	23
69	1.60	18	1.59	19	1.37	31
393	1.58	19	1.66	17	2.49	8
51	1.53	20	1.52	21	1.29	34

Table 5. Breeding values for the best 20 animals from sheep population for prolificacy

ANIMAL	BV-BLUP	Rank-BLUP	BV-BLP	Rank-BLP	BV-OP	Rank-OP
221	0.2603	1	0.2615	1	0.2016	10
219	0.2533	2	0.2542	2	0.2038	7
253	0.2510	3	0.2524	3	0.2014	11
31	0.2352	4	0.2320	5	0.1923	14
211	0.2350	5	0.2363	4	0.1681	38
235	0.2350	6	0.2363	4	0.1681	38
362	0.2216	7	0.2179	6	0.1791	24
271	0.2122	8	0.2134	7	0.2134	5
284	0.2122	8	0.2134	7	0.2134	5
32	0.2107	9	0.2078	8	0.1440	65
81	0.2049	10	0.2004	10	0.2021	9
327	0.2032	11	0.1968	13	0.1856	17
168	0.1988	12	0.2019	9	0.1729	30
249	0.1972	13	0.1982	11	0.2211	1
140	0.1960	14	0.1957	14	0.1225	82
258	0.1955	15	0.1971	12	0.2184	3
53	0.1932	16	0.1893	16	0.1497	56
88	0.1932	16	0.1893	16	0.1497	56
199	0.1897	17	0.1909	15	0.1762	27
365	0.1851	18	0.1616	27	0.1041	87

In the Tables 2, 3, 4 and 5 were shown the breeding values for the best 20 animals from sheep population for amount of milk, amount of fat, amount of protein, and prolificacy, for three selection methods, from the point of view of breeding value, selection based on the BLUP - Individual Animal Model methodology, selection based on the BLP method (Selection Indices) and selection based on own performance (OP). The breeding values of the best Palas sheep for amount of milk in our study the BLUP methodology and the BLP method and own performance, ranged from 12.90 to 26.09 , 12.45 to 25.91 and 10.78 to 27.60 kg, respectively for fat ranged from 2.36 to 5.07, 2.28 to 5.00 and 2.37 to 5.80 kg, for milk protein ranged from 1.53 to 3.05, 1.52 to 3.03 and 1.29 to 3.19 kg, and for prolificacy ranged from 0.1851 to 0.2603, 0.1616 to 0.2615 and 0.1041 to 0.2016 kg respectively. Popa et al. (2020) found that the average estimate breeding value for daily milk yield (kg) for the best 18 Teleorman Black Head Sheep were ranged between 14.57 and 28.48 kg. In another study the breeding value of the best 10 Teleorman Black sheep for the number of born lambs ranged from 0.013 to 0.022 (Pelmuş et al., 2019). Based on the Best Linear Unbiased Prediction value, breeding values for Karadi ewes for daily milk supply, protein, and fat percentages varied from -10.5293 to 10.7504, -2.0546 to 2.0097%, and -1.7033 to 1.4067%, respectively (Hama Khan et al., 2019). In another study estimated breeding values and selection index for milk yield, fat yield, protein yield and prolificacy of the top 20 dairy ewes of the Gunson's dairy sheep flock were (-10.78 to 16.4 kg, -0.35 to 0.82 kg, -0.24 to 0.74 and -0.15 and +0.30 lambs) respectively and significantly ($P < 0.05$) these values are lower than the values we found in our study (Sutera, 2018). Breeding values have been recorded, the breeding value of the first 19 ewes for milk production ranged from 70 to 86 kg (Buzu, 2016).

Spearman Rank Correlation

Spearman Rank Correlation between couples: (BLUP and BLP), (BLUP and own performances), (BLP and own performances) for the amount of milk, milk fat, protein, and prolificacy (Table 6).

Table 6. The Spearman Rank Correlation between the three methods

Traits	BLUP and BLP	BLUP and Own Performances	BLP and Own Performances
Amount of Milk	0.998	0.89	0.899
Milk Fat	0.996	0.907	0.919
Milk Protein	0.998	0.89	0.897
Prolificacy	0.953	0.837	0.912

Table 6 shows rank correlations (Spearman) between the following pairs of methods: (BLUP and BLP), (BLUP and own performances), (BLP and own performances). The results obtained, in the order of the method couples, were the following: 0.998, 0.89 and 0.899, for the amount of milk; 0.996, 0.907 and 0.919, for the amount of fat in milk and 0.998, 0.89 and 0.897, for the amount of protein in milk. For prolificacy, the rank correlation values were 0.953, 0.837 and 0.912. These results show that there is a high degree of concordance between the three selection methods, suggesting that any of the three methods can be used for selection candidates. Also, for each individual trait, the highest agreement was achieved between the BLUP and BLP methods (0.998, 0.996, 0.998 and 0.953), which can be explained by the proximity between the two calculation methods, from the point of view of the calculation methodology.

CONCLUSIONS

Heritability plays a pivotal role in predicting breeding values and advancing genetic progress. Understanding the heritability and genetic correlations of the traits under consideration in the breeding objective and selection index is crucial for accurate breeding value estimation.

Heritability estimates for the four examined traits were determined using the REML method within an animal model. These estimates were as follows: 0.197 ± 0.263, 0.209 ± 0.278, 0.263 ± 0.347 and 0.235 ± 0.311. These findings emphasize that these traits exhibit an intermediate level of genetic determinism. Genetic correlations between milk components and prolificacy to be positive and significant, which may have important implications in the selection of animals for genetic improvement.

By conducting selection for sheep using three distinct approaches and subsequently comparing them-namely, selection based on individual performance, selection based on the Best Linear Unbiased Prediction (BLP) method (Selection Indices), and selection based on the BLUP - Individual Animal Model methodology-we observed a high degree of agreement among the three selection methods. This suggests that any of these three methods could be effectively employed for candidate selection, the breeding goal should be to improve milk performance, particularly milk quality, taking into account prolificacy.as indicated by the strong Spearman's rank correlation between the pairs identified in this study.

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EVALUATING THE CURRENT IMPROVEMENT STAGE OF THE PREDOMINANT SKINS MODELING OBTAINED FROM KARAKUL OF BOTOSANI BREED

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Abstract

The purpose of the research was to carry out a real analysis of the degree of improvement for the traits that influence the quality of skins obtained from Karakul of Botosani lambs. The biological material was represented by lambs of both sexes obtained from adult sheep belonging to the respective breed. In order to evaluate the improvement of the modeling degree, assessments were made on several generations of lambs, starting with those from 2007 when the current Improvement Program is applied, those from 2015, 2020 and respectively from 2022. The working methods used are accepted by the experimental technique and the evaluation of the type of model was made based on the technical norms and in which it is specified that 50 points are awarded for very good modeling; for good modeling, only 25 are assigned, and for situations in which the modeling is satisfactory, only 10 points are given. Following the statistical processing of the data, it is found that the improvement process is more advanced in black variety lambs because an average score of 32.38 was obtained and from the total number of lambs evaluated in 2022, the proportion of those that were also associated with a valuable modeling was 66.8%.

Key words: *Botosani Karakul, color variety, pelts, skin.*

INTRODUCTION

The modeling is an overall property rendered by the placement of the curls between them and their distribution on the skins surface. This characteristic is primarily dependent on the clarity, contouring and relief of curls with the same rolling direction and with a similar degree of closure.

This character is determined in expression by the arrangement of the majority of curls on the surface of the skin. Ideally, on the surface of the skin there should be a uniformity of the curls in terms of length, width and height, but also in terms of their position on the surface of the skin.

That is why breeding work is a long-term process, and in each generation the genetic gain obtained can be very small. In reality the variability is very high because all of this is the result of complex interactions between several characteristics that contribute positively or negatively to obtaining a certain model (Anonymous, 1982, cited by Greeff et al., 1993; Schoeman, 1992).

The complexity of improving the traits related to skin for this characteristic is also attributed to the fact that the overall curling, along with other basic features of the curl, is influenced by the characteristics of the skin and hair follicles (Nel, 1965; Hugo, 1982; Dreyer et al., 1983; Ylmaz et al. 2013).

Analyzing this aspect, Hornitscek (1938), cited by Schoeman in 1998, found great differences in the follicular distribution and in the way the curls are arranged on the surface of the skin and, implicitly, in the pattern described by them.

In the case of selection applied to the brown and grayish variety the overall quality of the skins depends on some additional characteristics becoming important, while others, such as the predominant pattern, become less important (Gouws, 1974).

Lourens et al. (1999) demonstrated the importance of directing mating activities to ensure an intermediate color that promotes expression in the new genotype, as well as the desired pattern and fiber quality contributing to a better expression of the aesthetic and commercial value of the skins.

Other studies on light varieties demonstrate that while light shades have a weaker expression for pattern type, they are associated with better fiber quality. Other studies suggest that darker shades give more clearly defined patterns even when fiber quality is poorer.

MATERIALS AND METHODS

The biological material subjected to research belongs to several color varieties within the Karakul of Botoșani breed. All lambs evaluated are of known origin and are subject to production performance control, respectively that based on the evaluation in the PP system (own performance) and the control based on the OP type evaluation (origin and productivity).
Through applied research, the aim was to conduct a real analysis of the stage of improvement in the curling quality of Karakul Botoșani Sheep Breed lambs. Considering that the improvement process takes time, and the effect of selection is quantified at a low level in each generation, to objectively assess the degree of improvement for that trait, the values obtained in the evaluation season of 2022 were compared with the performances achieved in other seasons, namely 2007, 2015, and 2020.
Given that the quality of curling represents a complex trait, multiple features, including the pattern described by the arrangement of the curls on the skin surface, were assessed in each evaluation season. In essence, the purpose of the research was to conduct a current and objective analysis of the level at which the improvement process stands for the trait represented by the arrangement of the majority of curls on the skin surface and the pattern they exhibit through this positioning.
In order to obtain real data on the objectives analyzed during the research, several activities accepted by the experimental technique and suitable for the production of skins were carried out.
The working method used to assess the sought-after characters was based on the technical norms specified in Section 1.4 and 1.5 of MADR Order no. 22/20.01.2006, published in the Official Gazette of Romania no. 146 of 15.02.2006, which stipulates the aspects on the basis of which the Official Control of the production of skins is carried out in Karakul lambs of the Botosani and Metis breeds. During the assessment of the quality of the skins, the shaping of the curls can be very good and 50 points are awarded; for good modeling only 25 points are awarded and for situations where the modeling is satisfactory only 10 points are awarded.
In each season all assessments were performed by a minimum of two experienced technicians. If clear differences of opinion were recorded, each aspect invoked was discussed in detail until a consensus was reached.
The statistical processing of the data was based on the use of the S.A.V.C. computer program (Statistics Analysis of Variance and Covariance 2003). To test the statistical significance of the differences between the mean values of the studied parameters as well as the correlations between them, the Analysis of Variables (ANOVA Single Factor) and Pearson Correlation algorithms were used, both included in the computer program used.

RESULTS AND DISCUSSIONS

The modeling of the curl is given by the curls arrangement on the skin surface but also by the position of some in relation to others. It is a character under the direct influence of several factors, the most important of which are: the way the fibers are positioned in the curl, the degree of closure of the curls, the thickness, the direction of rolling as well as the shape, size, uniformity and distance between the curls.
In the qualitative evaluation of skins as modeling types, the most common are:
- lyre - when the curls are arranged perpendicular to the spine and with the sides oblique or forward;
- Christmas tree shape (when the curls are arranged on the sides, being quite straight, oriented backwards and with the longitudinal axis of the curls oblique to the upper line);
- mixed modeling or nut core is encountered when the curls have undefined arrangement and orientation between them, sinuous and with different directions (they are acceptable only if the clarity of the curls is good);
- rosette shape (when the curls diverge from a common point in all directions);

– wild modeling is when curls of different shapes are found on the surface of the skins, placed irregularly (they are worthless).

Modeling is assessed by examining several groups of curls, from different regions, and the qualifications are given as appropriate: very good, good, satisfactory, poor.

When the distance between the curls is small, the delimitation given by their contour is not clearly highlighted and, consequently, the modeling will be poor.

As the most common types of modeling, the skins obtained from lambs of the Karakul of Botosani breed are in the shape of a lyre, with fir, rosette, walnut core, mixed.

Although there is also a great variability of this character, the most appreciated are the pelts that express a correct contour of the curls, with a frequent arrangement in orderly formations arranged concentrically or parallel and with an intense luster.

For the black variety, after the evaluation of this character, a higher score was obtained compared to the one obtained for other color varieties, the average score being 32.38 points (Table 1). This level indicates that improvement must be intensified because the current expression of this character is 17.62 points away from the maximum score allowed in the evaluation.

However, obtaining an average score of 32.38 ± 0.53 indicates that the improvement process in this variety is at a higher level compared to other types of color. Also, from the data analysis it can be seen that in the black variety the proportion of lambs that obtained the maximum score in the analysis of the pattern type registered an increase from 19.60% in 2007 to 21.28% in the generation of the evaluation season of 2022. Also for this variety, it should be highlighted that the analysis of the predominant model recorded in the evaluation of this trait in lambs belonging to the generation of 2022 at a proportion of 66.8% of the total of 555 evaluated lambs shows that the curls have a predominantly concentric and parallel arrangement, an aspect that claims that improvement is proceeding in the desired direction and at a high degree of intensity. In research carried out on lambs belonging to the black variety from line 5 and 1557 of the same breed, Marin et al. (1974 and 1977) found that modeling was very good and good in about 70% of the population analyzed. Later, in 1977, the same group of authors found that in 71.10% of the lambs belonging to this breed, the modeling was good to very good, in another proportion of 24.60% it was of medium type and in 4.30% it was satisfactory.

In other researches carried out more recently, it is found that the improvement of this trait is increasing, positive and supported not only by the selection intensity but also by the mating management plan used in each breeding season (Buzu, 2023; Pascal, 2011; Nechifor, 2017; Nechifor et al., 2022).

Comparing these results with those determined in the current generations could be considered as inferior, and in this case, instead of genetic progress, there was a regression, which is not true because all the results presented in Table 1 refer only to individuals with a modeling of the desired type and only to those who received maximum points in the evaluation of this character.

Also, in the assessment of the current state of improvement, the information regarding the frequency of individuals in the population who also had a well-expressed modeling can be used (Figure 1). In the case of this variety, out of the total number of evaluated lambs, a concentric-parallel pattern was found at 66.80%. But the fact that individuals with a less desirable phenotypic expression, of mixed and undefined type, represent a total proportion of 2.8% indicates a good genetic consolidation but also a good degree of hereditary transmission and a higher efficiency of the selection process.

In the grayish variety, the average score was 27.16 ± 0.21 and the difference of 5.22 points compared to the average score determined in the black variety is significant for $p<0.01$ (Figure 1 and Table 2).

The improvement of modeling and the definition of a better contoured pattern, which participates in a substantial increase in the aesthetic and commercial value of skins, is a process in which genetic progress is quite difficult to achieve. This aspect confirmed by the fact that the proportion of lambs that obtained a higher score in the modeling evaluation increased by only 2.78% in 2015 compared to 2007 and by only 1.43% in 2022 compared to 2015.

Table 1. Statistical Parameters for Curl Modeling Types

Color variety	Total evaluated lambs (n)				$\overline{X} \pm s_{\bar{x}}$	V%	Very good modeling (%)				Predominant pattern in lambs evaluated in 2022 (%)			
	2007	2015	2020	2022			2007	2015	2020	2022	cp*	p*	m*	u*
Black	774	815	885	555	32.38±0.53	35.76	19.60	19.85	20.44	21.28	66.8	30.4	2.5	0.3
Grayish	348	410	422	440	27.16±0.21	27.56	10.85	12.20	13.50	13.63	68.7	30.1	0.9	0.3
Brown	225	185	198	210	26.38±0.39	30.88	9.13	10.36	11.07	8.75	19.9	72.1	7.1	0.9
Grey	103	122	130	155	27.63±0.42	35.32	11.25	12.32	12.85	13.05	18.7	72.3	7.7	1.3
Pink	230	198	201	230	26.41±1.13	43.75	9.05	9.26	9.52	9.55	16.1	59.2	22	2.7

*: cp = concentric-parallel; p = parallel; a = mixed; n = undefined

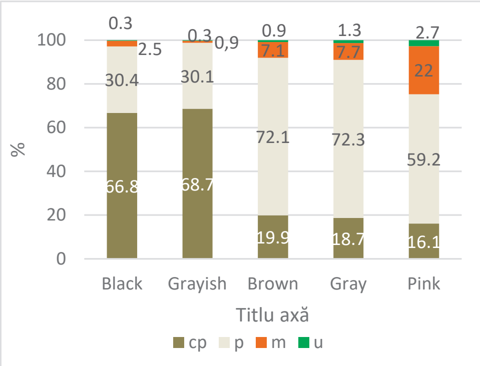

Figure 1. Model Type Quality Classes 2022 Generation (%)

Table 2. The Difference and Significance of Difference for Curl Shaping

Caracter 1	Caracter 2	Mean difference	The meaning of the difference	Threshold of significance
Gray	Grayish	0.47	ns	-
Gray	Brown	1.25	ns	-
Gray	Black	4.75	**	0.01
Gray	Pink	1.22	ns	-
Pink	Grayish	0.76	ns	-
Pink	Brown	0.03	ns	-
Pink	Black	5.98	**	0.01
Black	Grayish	5.22	**	0.01
Black	Brown	6.01	**	0.01

The analysis of this trait indicates a higher incidence of the expression of a pattern accepted in breeding because the proportion of those with a concentric-parallel pattern is higher by more than 50% compared to other types of patterning, and the presence of a lower proportion of 1.2% of lambs with undesirable patterning highlights the effectiveness of selection.

At brown lambs, an average score of 26.38 was obtained for the assessment of the type of model. The difference compared to the average values obtained for the other four varieties is not statistically significant. The analysis of the proportion of lambs that were appreciated with maximum points shows an increase in 2015 compared to 2005 as the proportion of lambs with higher scores was 11.07 in 2015 compared to 10.36 in 2005.

In the generation of brown lambs evaluated in 2022, it is observed that compared to 2020, the proportion of lambs that obtained a higher score is reduced from 11.07% to 8.75% (Figure 2).

For this variety, the continuation of the improvement of this trait requires a reevaluation of the selection criteria and increased attention in directing the pairings so that the predominant model manifested is predominantly the concentric-parallel type.

When evaluating the type and model of the curl in lambs belonging to the grey variety, the average score obtained in lambs evaluated in 2022 was 27.63 ± 0.42 points. Based on this score, we can appreciate that the arrangement of the curls on the surface of the skin is uneven and does not express a clear and well-defined outline. Regarding the number of lambs that obtained a higher score, their proportion increases from 11.25% in 2007 to 13.05% in 2022.

When evaluating the type of modeling in lambs belonging to the pink variety, the proportion of those who obtained a higher score is kept constant and has not exceeded 10% since the year in which the Improvement Program of the Karakul Botosani sheep breeding breed was developed and applied, respectively 2007.

Based on these data, we can appreciate the fact that in lambs of the sour and pink variety, although the process of improvement of the characters on which the quality of the skins depends is in the initial stages, the average score higher than 25 points shows a good expression of the modeling in the current generations.

The prevailing model in both varieties is represented by the parallel arrangement of the curls, however, the objective of the improvement is represented by moving the frequency in the area of the desired type.

Between the average score obtained for the variety grey x black and pink x black the differences are significant for $p<0.01$, indicating a high degree of confidence.

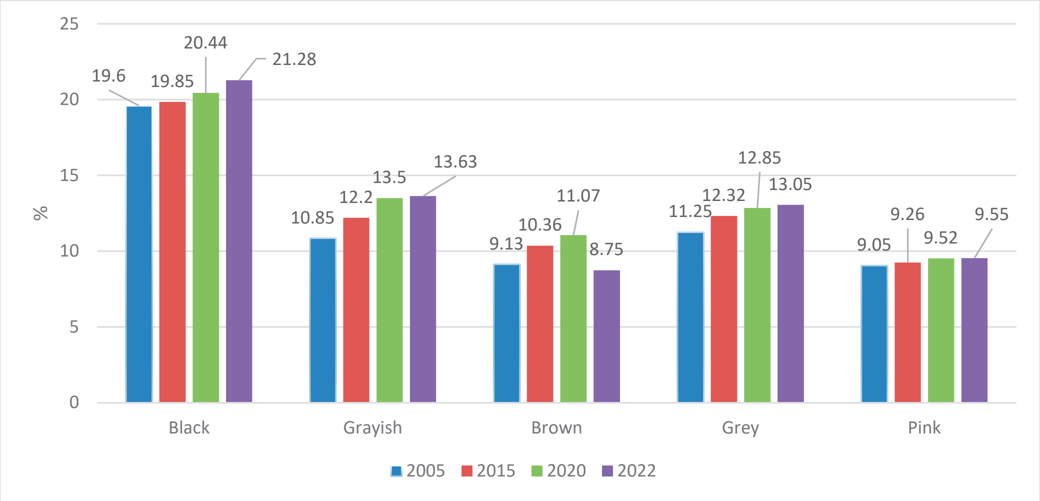

Figure 2. Proportion of lambs that had very good shaping

CONCLUSIONS

Following the evaluation of the degree of modeling, a great variability of this character is found and in order to continue the improvement process, the activities that can induce a correct contour of the curls, an intense shine and an arrangement of the curls in concentric or parallel pattern must be intensified.
For the black variety, improvement efforts to enhance modeling need to be intensified as the average score is slightly above 30 points. By increasing rigor in the selection process, the proportion of lambs with undesirable expression is expected to be well below the current 20%.
When assessing the degree of modeling in the Grayish variety, the score based on the average score obtained, we can say that the applied selection and management of breeders contribute to increasing the proportion of lambs that obtain a higher score.
Based on these data, it can be recommended that the selection at grayish should focus on intermediate color types, because this type is associated with an acceptable model but also with a better fiber quality.

The analysis of the proportion represented by the brown lambs that were appreciated with the maximum points shows a decrease, in 2015 compared to 2007, at 0.38%, the probable cause being represented by certain inconsistencies between the criteria that were the basis for drawing up the routing list of pairings.
For the gray and pink varieties, the results show an insignificant increase in lambs that had a score characteristic of the desired pattern when evaluating the type of pattern.

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ESTIMATION THE GENETIC PARAMETERS FOR GROWTH TRAITS IN ABERDEEN ANGUS BREED

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Abstract

The objective of this study was to estimate the breeding values and genetic parameters for birth weight and weaning weight in Aberdeen Angus cattle breed with maternal animal model. Data consisted of records of 1206 calves of Aberdeen Angus breed from Aberdeen Angus Association Romania. The direct breeding values for birth weight were in the population between -12.8 and 21.93 and for weaning weight from -82.68 to 155.10. The direct breeding values for calves with records for birth weight were from -8.87 to 16.077 and for weaning weight from -71.51 to 150.26. The maternal breeding value for birth weight ranged between -2.772 and 3.388 and for weaning weight from -38.273 and 49.693 in the population. The maternal breeding values for calves with records for birth weight ranged between -2.206 and 1.668 and for weaning weight were from -25.824 and 27.906. The direct heritability for birth weight was 0.266 and for weaning weight was 0.217. The maternal heritability was 0.048 for birth weight and 0.081 for weaning weight. The total heritability was 0.24 for birth weight and 0.20 for weaning weight.

Key words: *breeding value, genetic parameters, maternal animal model.*

INTRODUCTION

The birth weight and the weaning weight are important traits in beef cattle. Aberdeen Angus is a breed from Scotland used for beef production. The breeding program of Aberdeen Angus breed in Romania have the objective the improvement of the traits for meat production and the reproduction and functional traits. Aberdeen Angus cows breed is resistant to environment conditions, adaptable, mature extremely early and the cows have a high carcass yield with marbled meat. This breed has a good meat quality. The females of Aberdeen Angus breed calve easily and have good ability to rear the calves. The maternal animal model was used for genetic evaluation of beef cattle. The maternal animal model was used in studies by different authors. The aim of this study was to estimate the breeding values and genetic parameters for birth weight and weight at 6 months in Aberdeen Angus breed using a maternal animal model for the selection of the cows. The growth traits in beef cattle are still the base information in genetic evaluation (Otto et al., 2021). Even if in the world wide the Angus breed is well studied and BLUP methodology is applied to different animal models (Boddhireddy et al., 2014), in Romania, Angus breed is just at the beginning of breeding programs and genetic evaluations (Madescu et al., 2022; Gociman et al., 2019). Because in our study we discuss of a population of Angus cattle of the Romanian territory, it is mandatory to have local estimation of genetic parameters that will be particularly to our studied population.

MATERIALS AND METHODS

The data were from 1206 Aberdeen Angus calves. The pedigree consisted in 2563 animals: 1206 calves with performances, 1203 dams and 154 sires. The calves were born in 2021 and 2022 years, even in the data set were two years of calving, it was no difference between years and it was not used as a fixed effect in the biometrical model. The data were from Aberdeen Angus Association Romania. The cows' data were from 169 farms and herd was used in the model as an fixed effect.

The weaning weights were corrected for standardized age.

The usual method for calculating standard age weight is based on determining average daily gain between two weight recordings; then, assuming growth to be linear between the recordings, estimate live weight increase from the day of first recording and reference day and add it to weight on first recording. It is preferable that the age to which weight is being adjusted occurs between two successive recordings. If this is not possible, an extrapolation is possible if age at last recording falls within a specific time interval from the standard age. The time interval has to be determined by each recording organization based on recording frequencies (ICAR, Section 3-Beef cattle).

Where with the exception of birth weight, there is only one weight record available after birth:
- let AR be reference age;
- let WR be weight at reference age;
- let DB be birth date let;
- Dt be recording date t;
- let WB be birth weight;
- let Wt be recorded weight at date t;
- let At be age of animal at recording date (= Dt - DB).

If AR < At
then
WR = [(Wt - WB)/ At]*AR + WB
If AR > At
then WR = {[(Wt - WB)/ At]*(AR - At)]} – Wt

For estimate the genetic parameters was used the maternal animal model. For analyze the data was used the R software, the script was realized by Grosu (Grosu & Oltenacu, 2005).
The model was (Mrode & Thompson, 2005):

y = Xb+Za+Wm+Spe+e
where:
y = the vector of performances;
b = the vector of the fixed effects, in our study was the sex of calves and the herd;
a = the vector of the random animal effects;
m= the vector of the random maternal genetic effects;
pe = the vector of the permanent environmental effects and
e = the vector of the random residual effects.
X, Z, W and *S* are the incidence matrices referring to animal performance, to the fixed effects, to the direct effects, to the maternal effects and to the permanent environmental effects.
It is assumed that:

$$\mathrm{var}\begin{bmatrix} a \\ m \\ pe \\ e \end{bmatrix} = \begin{bmatrix} \sigma_a^2 A & \sigma_{am} A & 0 & 0 \\ \sigma_{am} A & \sigma_m^2 A & 0 & 0 \\ 0 & 0 & I\sigma_{pe}^2 & 0 \\ 0 & 0 & 0 & I\sigma_e^2 \end{bmatrix}$$

where:
A is the kinship matrix between animals;
I is the identity matrix;
σ^2_a is the additive genetic variance for the direct effects;
σ^2_m, the additive genetic variance for the maternal effects;
σ_{am}, the additive genetic covariance between the direct and maternal effects;
σ^2_{pe}, is the variance due the permanent environmental effects;
σ^2_e, is the variance of the residual error.

The genetic parameters were estimated based on the next formulas:

- the direct heritability:

$$h_a^2 = \sigma^2_a / \sigma_p^2$$

where σ^2_a and σ_p^2 is the genetic and phenotypic variance;

- the maternal heritability:

$$h_m^2 = \sigma^2_m / \sigma_p^2$$

where σ_{am} direct is maternal additive genetic covariance;

- the total heritability (Wilham et al., 1972):

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

- the genetic correlation between the direct and maternal effects:

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

The error of heritability (Hoj-Edwards, 2017):

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

where:

$$\frac{\partial h^2}{\partial \sigma_g^2} = \frac{\sigma_e^2}{(\sigma_g^2+\sigma_e^2)^2}\,;\ \frac{\partial h^2}{\partial \sigma_e^2} = \frac{-\sigma_g^2}{(\sigma_g^2+\sigma_e^2)^2}\,;\ \rho_{g,e} se_g se_e = cov(\hat{\sigma}_g^2, \hat{\sigma}_e^2)$$

RESULTS AND DISCUSSIONS

The average performances for growth traits for Aberdeen Angus cattle are presented in Table 1.

Table 1. The average performances for growth traits

No.	Birth weight	Weaning weight
Mean	29.954	219.007
Standard error	0.142	1.272
Median	29	215.5
Mode	30	225
Standard deviation	4.952	44.179
Sample variance	24.524	1951.863
Kurtosis	1.432	2.512
Skewness	0.848	0.808
Range	31	380.45
Minimum	19	78.12
Maximum	50	458.57
Sum	36125	264123.49
Count	1206	1206
Confidence level	0.279	2.495
Coefficient of variation (%)	16.53	20.17

The mean of birth weight in our study was lower than the mean reported by Nikolov and Karamfilov (2020) in Aberdeen Angus breed 31.6 kg and higher that the mean reported by Jakubec et al. (2003) 29.22 kg in Aberdeen Angus from Czech Republic and Kolisnyk et al. (2018) in Ukraine, 26.5 kg for the females and 29.4 kg for the males. The weaning weight (at 200 days) in our study was higher than the weaning weight at 210 days, 204 kg in Aberdeen Angus from organic farm from Bulgaria.

Jakubec et al. (2003) obtained the mean for weight at 210 days 241.41 kg and 379.50 at 365 days in Aberdeen Angus cattle from Czech Republic. Meyer (1995) reported the birth weight 34.07 in Aberdeen Angus calves from New Zealand and 33.27 kg for calves from Australia and the weaning weight 216.8 kg, respectively 233.1 and the weight at 365 days 285.8 kg, respectively 337.5. Crawford et al. (2016) reported the birth weight 36.2 kg, the weaning weight 213.5 kg and yearling weight 345.6 kg in Angus breed.

For the fixed factors in the model were ranged from -9.3 to 13.61 for birth weight and from -86.66 to 96.07 for weaning weight when we discuss about the influence of herd as a fixed factor. Regarding the sex factor it was 29.29 in birth weight and 212.23 in weaning weight for females and for males it was 29.97 for birth weight and 224.18 for weaning weight.

The factors which influenced the weight of cattle are: the breed, herd, sex, year, age of cow, season and month of birth. The direct and maternal breeding values for the best animals are presented in the Table 2.

Table 2. The direct and maternal breeding values of the 10 best Aberdeen Angus cattle for birth weight and the weaning weight

No.	The direct breeding values for the birth weight	The direct breeding values for the weaning weight	The maternal breeding values for birth weight	The maternal breeding values for weaning weight
1.	17.8726	89.6712	3.0859	53.9754
2.	13.5882	63.8356	2.7191	38.2261
3.	13.5372	59.8817	2.1664	30.9036
4.	13.5197	56.6923	1.7084	22.8797
5.	13.3831	46.5922	1.6791	22.5334
6.	13.1785	45.8782	1.6075	22.3953
7.	12.8879	45.6202	1.5795	21.0499
8.	12.839	44.7397	1.5617	20.9878
9.	12.2577	42.0329	1.5083	20.7223
10.	12.2359	42.0271	1.4701	19.7131

In Table 3 were the direct and maternal breeding values for calves with records. Duchacek et al. (2011) estimated the breeding values for weaning weight in Aberdeen Angus from Czeck Republic.

Table 3. The direct and maternal breeding values of the 10 best Aberdeen Angus calves with records for birth weight and the weaning weight

No.	The direct breeding values for the birth weight	The direct breeding values for the weaning weight	The maternal breeding values for birth weight	The maternal breeding values for weaning weight
1.	13.5882	63.8356	2.7191	38.2261
2.	13.5372	59.8817	2.1664	30.9036
3.	13.5197	56.6923	1.7084	22.8797
4.	13.3831	46.5922	1.6791	22.5334
5.	13.1785	45.8782	1.6075	22.3953
6.	12.8879	45.6202	1.5795	21.0499
7.	12.839	44.7397	1.5617	20.9878
8.	12.2577	42.0329	1.5083	20.7223
9.	12.2359	42.0271	1.4701	19.7131
10.	11.3321	41.5604	1.4662	19.3915

The mean breeding values for direct effects ranged between 1.76 and 4.73 between the years 1997-2007. Duckacek et al. (2011) constated that the increased number of Aberdeen Angus cattle included in performance recording in the Czech Republic resulted in increased value of breeding value for direct effect for weight at 210 days of age.

The estimates of (co)variance components, direct heritability, maternal heritability, direct-maternal genetic correlation and fraction of total variance due to maternal permanent environmental effects for growth traits are shown in Table 4.

Table 4. Estimates of (co)variance components and genetic parameters for birth weight, weaning weight for Aberdeen Angus cattle breed

Item	Birth weight	Weaning weight
σ^2_a	6.39±0.260	506.873±20.65
σ^2_m	1.17±0.047	189.529±7.72
σ_{am}	-0.79	-90.712
σ^2_{pe}	14.98±0.610	1360.592±59.43
σ_e^2	0.65±0.026	179.674±7.31
σ_p^2	24,01±0.978	2327.381±94.81
c^2	0.623	0.584
$\sigma_{am}/\ \sigma_p^2$	-0.032	-0.038
h_a^2	0.266±0.106	0.217±0.046
h_m^2	0.048±0.055	0.081±0.053
r_{am}	-0.291	-0.292
h_T^2	0.240±0.108	0.200±0.048

σ^2_a direct additive genetic variance, σ^2_m maternal genetic variance, σ_{am} direct–maternal additive genetic covariance, σ^2_{pe} maternal permanent environmental variance, σ_e^2 residual variance, h_a^2 direct heritability, h_m^2 maternal heritability, $c^2 = \sigma_{pe}^2/\sigma_p^2$ ratio of maternal permanent environmental variance to phenotypic variance, σ_{am} / σ_p^2 covariance between direct and maternal effects as proportion to phenotypic variance, r_{am} genetic correlation between direct and maternal effects, h_T^2 total heritability.

The genetic parameters ranged in the values obtained in the literature. Meyer (1995) obtained the direct heritability for birth weight in Aberdeen Angus from New Zealand 0.286 and for Aberdeen Angus from Australia 0.379 and for weaning weight the heritability was 0.201 and respectively 0.230. The maternal heritability reported by Meyer (1995) was 0.096 for birth weight and 0.081 for weaning weight in Aberdeen Angus from New Zealand. The maternal heritability for Aberdeen Angus from Australia was 0.066 for birth weight and 0.084 for weaning weight. Crawford et al. (2016) reported the direct heritability for birth weight was 0.42 and maternal heritability was 0.14, for weaning weight, the direct heritability was 0.26 and maternal heritability was 0.23 and the direct heritability at 365 days 0.45 and maternal heritability 0.23. Costa et al. (2011) reported the direct heritability for weaning weight 0.44 and for yearling weight 0.43. The maternal heritability was 0.25 for weaning weight and 0.12 for yearling weight. Williams et al. (2012) obtained the heritability for weaning weight at low altitude 0.28 and 0.26 at high altitude in Angus breed. Boddhireddy et al. (2014) obtained the heritability for birth weight 0.42, for weaning weight 0.20 and for yearling weight 0.20 in Angus breed. Baccino et al. (2020) obtained the heritability for birth weight 0.25 and for weaning weight 0.16. Robinson et al. (1996) reported direct heritability for Australian Angus cattle 0.35 and maternal heritability 0.08 for birth weight, direct heritability for weaning weight 0.20 and maternal heritability 0.09 and for yearling weight direct heritability 0.24 and maternal heritability 0.06.

The covariance and the correlations between the direct and maternal genetic effects for birth weight and weaning weight were negative in our study. The genetic parameters from our study were influenced by the variable environmental factors due the different herds. Robinson et al. (1996) reported also the correlations between direct and maternal effects were large and negative. Gociman et al. (2019) reported that in 2019 were in the Aberdeen Angus Romanian Herdbook 45000 cattle in Romania and the breed was adapted well in the Romanian pedoclimate conditions. Table 2 shows the breeding values for all the cattle analyzed, not just the one with performance, and because of that we can observe that the best animal has an anormal high breeding value. The reason for that is because in real practice, the information from farmers are very different and we cand have a farm with 1 male that has 1 calf and that one it is very possible to be one of the best, and because of that, his sire will have a huge breeding values. On the opposite we can have a sire with multiple calves and his breeding value will be corrected based on much more information and for that reason the breeding value cand be smaller. In that way we can say that a breeding value of +21.93 can be accepted in the analyze but we do not recommend to farmers we promote that animal further to reproduction.

CONCLUSIONS

The direct breeding values of the best cows were between 15.18 and 21.93 kg for birth weight and between 82.49 and 155.10 for the weaning weight. The direct heritability for birth weight and weaning weight was moderate and the maternal heritability for these traits was low. Genetic parameters for birth weight show that these traits have huge variability, in specially when we look at the maternal heritability. One conclusion that can be obtained is that based on the birth weight breeding values is very difficult to have a valid selection decision and because of that is even more important to use the best biometrical model in the estimation of breeding values. Only in that way we can make objective decision regarding the genetic selection process. The most important conclusion and the aspect that offers the novelty characteristic of this work is the fact that the genetic parameters are, as mentioned in all breeding books, particular and characteristic of each individual population and each individual generation, and their estimation must be done either how many times there are changes in the structure of a cattle population. Moreover, the genetic parameters can be used in the breeding programs carried out on the territory of Romania.

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INFLUENCE OF PRODUCTION YEAR ON THE MILK PRODUCTIVITY IN EWES FROM THE BULGARIAN DAIRY SYNTHETIC POPULATION

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Abstract

The purpose of the study was to investigate the influence of production year on the milk yield per standard 120-day milking period of sheep from the Bulgarian Dairy Synthetic population in the flock of Agricultural Institute-Shumen. Milk productivity data of 2193 sheep of different ages for six production years, were analysed. For the individual years, the following were determined: duration of lactation and milking period, milk yield per milking period (TMM), average daily milk yield per milking period ($ADMY^{milking\ period}$) and milk yield per 120-day standard milking period (TMM120). The influence of the production year on milk yield per standard 120-day milking period was determined by the ANOVA model for one-way analysis of variance. The total milk productivity for the standard 120-day milking period of sheep in the individual production years was within 94.798-115.541 l, with the determined differences having a high degree of significance ($P \leq 0.001$). A highly significant effect of the production year factor on the milk yield per standard 120-day milking period per consecutive lactation was established.

***Key words**: Bulgarian Dairy Synthetic Population, milk productivity, production year, sheep.*

INTRODUCTION

Sheep, milk productivity is significantly affected by various genetic (intrinsic) and non-genetic (extrinsic) factors. Their impact is usually simultaneous, and it is difficult to determine the extent of their separate influence (Adamu, 2021; Ali et al., 2020; Alkass & Akreyi, 2016; Al-Najjar et al., 2022; Assan, 2020; Carta et al., 2009; Gonzalez-Ronquillo et al., 2021; Jawasreh & Khasawneh, 2007; Libis-Márta, et al., 2021; Oravcova et al., 2006, 2007; Pacinovski et al., 2012, 2016; Pulina et al., 2007; Robles Jimenez et al., 2020; Selvaggi et al., 2017). Dairy sheep breeding in Bulgaria has deep traditions and nowadays, dairy sheep make up about 75% of the total population and give the largest share of sheep breeding production. Commercial breeds for milk are represented, to the highest degree, by sheep from the Bulgarian Dairy Synthetic Population (BDSP), the Lacaune, Assaf and Avasi breeds. According to the data of the Executive Agency for Selection and Reproduction in Livestock Breeding, in 2023 the following are covered under selection control: 172861 sheep from BDSP; 17130 sheep of the Lacaune breed; 16652 sheep of the Assaf breed and 2592 sheep of the Awassi breed. The number of sheep from other local and imported dairy breeds is significantly lower. It is quite obvious that sheep from BDSP have a leading role in the production of milk, meat and derived products for feeding the population. The main goal of the selection is to increase milk productivity combined with good fertility. Although the animals have the potential for high milk yield (150-200l per milking period), the achieved results are unsatisfactory (Iliev et al., 2022; Ivanova, 2013; Slavova et al., 2015; Slavova & Stancheva 2023; Stancheva et al., 2014; 2018; 2021; 2022; Zhelyazkova et al., 2014). In all studies, the authors conclude that the realization of the genetic potential of BDSP sheep depends mostly on the provided conditions of nutrition, breeding, management of the production system. The production year, which largely takes into account the influence of so-called external factors (production system, rearing conditions, health status, nutrition, physiological condition of the animals, management and marketing strategy on the farm, specific daily animal care, etc.) probably has a significant effect on the dynamics of milk productivity and the realization of the genetic

potential of sheep. This also motivates our present study.
The purpose of the study was to determine the influence of the production year on the milk yield for a 120-day standard milking period of the ewes from the Bulgarian Dairy Synthetic population in the flock of the Agricultural Institute - Shumen.

MATERIALS AND METHODS

The study covered 2193 ewes from the Bulgarian Dairy Synthetic Population, bred at the Agricultural Institute - Shumen. The sheep were of different ages (from the 1st to the 7th lactation) and produced in the period 2015-2020. They are divided into 3 flocks and were raised on barn and pasture under a semi-intensive system. Animals designated for breeding are kept separately until they enter the main herd, at the age of 18 months. The animals were fed with their own feed. The lambing season usually takes place from the second half of November and ends by the middle of January. Milking is by machine in a milking parlor and twice a day after the lambs are weaned.

Figure 1. Monthly milk control in the flock of the Agricultural Institute - Shumen (own source)

Milk productivity

A total of 2193 milk yield records per milking period, average daily milk yield per milking period, length of lactation and milking period and 2010 milk yield records for 120-day standard milking period of ewes during the production years studied were analyzed. Milk yield data were obtained by measuring the amount of milk in liters milked during the milking period of the animals according to the AC method specified in the nomenclature of the International Animal Control Committee (ICAR). The first monthly controls were carried out in the months of December - February, and the last - in May and June. The milk yield of each sheep for the control day is the amount of milk in the morning individual control multiplied by the coefficient of the flock (K = morning + evening milk/morning milk). Milk yield per milking period (TMM) is the sum of the milk yield from the individual control periods of each ewe. The average daily milk yield for a milking period ($ADMY^{milking}$ period) is the amount of milk received per milking period divided by the duration of the milking period in days. Milk yield for a standard 120 day milking period (TMM120) is the average daily milk yield for a milking period multiplied by 120 days ($TMM120 = ADMY^{milking\ period} * 120$). For the individual production years, the average statistical parameters were established for: duration of lactation and milking period, milk yield per milking period (TMM), average daily milk yield per milking period ($ADMY^{milking\ period}$) and milk yield per standard 120-day milking period (TMM120) (total and after another lactation). Due to the relatively small number of ewes in the 6th and 7th lactation, their milk yield data for a standard 120-day milking period were grouped into one category 5+, so that 5 levels were obtained for TMM120 per consecutive lactation. The obtained results were processed using the software Statistica. The influence of the production year on the milk yield for a 120-day standard milking period (total and consecutive lactation) was determined by the one-way analysis of variance ANOVA model.

RESULTS AND DISCUSSIONS

The statistical parameters for the investigated signs of the total milk productivity of sheep for the production years 2015-2020 are shown in Table 1. The duration of the lactation and milking period is an important systemic source for environmental changes and variability of milk productivity. The average duration of the mammary and milking period in the studied years varies from 52 to 59 days for the former, and is within 131-162 days for the latter. The significant differences between the established minimum and maximum values are indicative of the need for changes in the management of the production system on the farm. In this direction, the reduction of the lactation period is a significant reserve for extending the milking period and increasing the amount of milk obtained for sale (Simeonov et al., 2012; Ivanova et al., 2015; Mavrovska, 2015; Stancheva et al., 2018; Miteva, 2022). Milk yield per milking period increased to 139.681 l in 2019 and decreased to 120.559 l in the following year, 2020. The values of the average daily milk yield for the milking period gradually increased until the year 2017 ($ADMY^{milking}$ period 0.935 l), after which they significantly decreased. As unfavorable, we can point out the production years 2015, 2016 and 2020, where the values for milk yield per milking period (103.625 l, 119.657 l and 120.559 l) and average daily milk yield per milking period (0.791 l, 0.838 l and 0.824 l) are the lowest.

Table 2 shows the total average values of milk yield for a 120-day standard milking period (TMM120) for the period 2015-2020, depending on the production year and the sequence of lactations. For the study period, the average value of milk yield per 120-day standard milking period (TMM120) was 103.379 l. The level achieved is well below the set breeding target of 150 l and below, the minimum selection limit for the Elite class of the population (105 l). The high variability of the trait is illustrated by the standard deviation values (SD 30.34).

Depending on the production year, the milk yield for a 120-day standard milking period increased to 115.541 l in 2017, after which it significantly decreased. The lowest, and with values below the general average, is the milk yield of the sheep produced in 2015, 2016 and 2020 (94.798 l, 99.624 l and 98.641 l). In our opinion, these results do not reflect the genetic endowments of the animals, but rather are due to various, non-biological factors such as rearing conditions, unbalanced nutrition in relation to the physiological state of the animals, daily care and health status of the animals, gaps in husbandry technology and non-genetic interactions. Analysis of variance reported a highly reliable effect of year of production on total milk yield of ewes over a 120-day standard milking period. A reliable effect of the year of production was found by Zhelyazkova et al. (2014) in SPBM sheep bred in two private flocks, Al-Najjar et al. (2021), Jawasreh and Khasawneh (2007), Pacinovski et al. (2016), Üstüner and Mustafa (2013) on Awassi sheep bred in Jordan, Turkey and its crosses in Macedonia, Selvaggi et al. (2017) on three Italian sheep breeds. It is known that the milk productivity of ewes increases with the succession of lactations (Hinkovski et al., 2008; Ivanova, 2013; Iliev et al., 2021; Kasap et al., 2019; Miteva, 2022; Slavova et al., 2015; Robles Jimenez et al., 2020; Selvaggi et al., 2017; Sezenler et al. 2016). Our obtained results are not exactly like that (Table 2). The average milk yield values for a 120-day standard milking period are higher than or close to the total flock average by the 4th lactation as for ewes at 1st lactation are the highest (107.747 l). It can be seen that the animals show their genetic potential for high milk yield already in their 1st lactation. Similar results were obtained by Pollott and Gootwine (2004) in sheep of the Assaf breed and Elvira et al., (2012) in the Lacaune breed. In the next two lactations, the mean values of the trait decrease, but the milk yield achieved for a 120-day standard milking period still approaches the population Elite class limit (105 l) in ewes of the 4th lactation (103.129 l). We found significantly lower milk productivity in the animals of the 5th and more lactations, as the milk yield for a 120-day standard milking period (90.176 l) does not cover the selection limits for the 1st class of the population (95 l). The analysis of variance here also reports a highly reliable effect of the production year on the milk productivity of the sheep for a 120-day standard milking period per consecutive lactation.

Table 3 shows the total average values of milk yield for a 120-day standard milking period (TMM120) by consecutive lactation, during individual production years. The results show that in the 1st lactation, the milk yield for a 120-day standard milking period increases until 2018-2019 years, after which it significantly decreases. The highest, and with values exceeding the selection limits for the Elite class, is the milk yield of sheep in the years 2018, 2017, 2019 and 2020 (115.413 liters, 114.851 liters, 114.152 liters and 107.427 liters). The milk yield of 2nd lactation ewes does not increase significantly compared to the milk productivity of 1st lactation ewes. The highest, and with values exceeding the selection limits for the Elite class, is the milk yield for a 120-day standard milking period in the years 2017, 2018 and 2019 (118.545 l, 115.306 l and 107.511 l). Although the milk yield of the sheep in 2015, 2016 and 2020 was lower, it still approached the Elite class limit of the population in 2020 (103.408 l) and exceeded the requirements for I class in the animals produced in 2015 and 2016 (98.501 l and 100.319 l).

Table 1. Statistical parameters for some traits of the milk productivity by production year

Traits / Year	n	x	SD	Min.	Max.
year 2015					
Suckling period (days)	365	53	8.01	26	77
Milking period (days)	365	131	12.20	67	141
TMM (l)	365	103.63	31.27	25.625	219.921
ADMY milking period (l)	365	0.791	0.22	0.279	1.560
year 2016					
Suckling period (days)	373	54	13.50	7	114
Milking period (days)	373	143	24.00	42	170
TMM (l)	373	119.657	41.17	10.385	266.896
ADMY milking period (l)	373	0.838	0.27	0.247	1.822
year 2017					
Suckling period (days)	370	58	9.71	24	94
Milking period (days)	370	134	20.97	42	192
TMM (l)	370	127.36	44.54	20.536	269.171
ADMY milking period (l)	370	0.935	0.27	0.298	1.831
year 2018					
Suckling period (days)	353	52	12.32	18	97
Milking period (days)	353	148	19.76	58	182
TMM (l)	353	134.86	48.61	25.748	308.611
ADMY milking period (l)	353	0.907	0.29	0.303	1.868
year 2019					
Suckling period (days)	384	52	54.00	5	80
Milking period (days)	384	162	17.25	58	213
TMM (l)	384	139.68	46.11	31.238	258.863
ADMY milking period (l)	384	0.854	0.25	0.336	1.523
year 2020					
Suckling period (days)	348	59	12.61	7	85
Milking period (days)	348	146	17.39	57	193
TMM (l)	348	120.56	38.06	21.684	261.041
ADMY milking period (l)	348	0.824	0.24	0.380	1.729

On the next 3rd lactation, a drop in milk productivity was observed, except for the milk yield of the ewes lactating in 2018 (132.263 liters), which significantly exceeded the general average for the flock and the selection limits for the Elite class of the population. For the animals that produced in the remaining production years, the milk yield for a 120-day standard milking period is within the limits of 90.188 liters in 2015 to 108.254 liters in 2019. The decrease in milk productivity continues in the next 2 lactations. In the ewes of the 4th lactation, the highest, and with values exceeding or equal to the selection limits for the Elite class, is the milk yield for a 120-day standard milking period in the years 2018 and 2017 (122.330 liters and 105.905 liters). During the rest of the production years, the milk productivity of the animals meets the requirements for the 1st class of the population. We found significantly lower milk productivity in the animals of the 5th and more lactations, as the milk yield of the sheep for a 120-day standard milking period exceeded the selection limits for the 1st class of the population, only in 2016 and 2017 (99.572 liters and 103.431 liters). The established differences in the milk productivity of sheep during individual production years have a high degree of reliability for all lactations ($P \leq 0.001$).

Table 2. Overall mean and analysis of variance for a milk yield per standard 120-day milking period, (l) (period 2015-2020 years)

Variable	**Milk yield per standart 120-day milking period (TMM^{120}), (l)**			***P*-value**
	n	***x***	***SD***	
TMM^{120}, total	2010	103.379	30.34	
TMM^{120} by production year				
year 2015	347	94.798	25.64	**0.000000**
year 2016	334	99.624	28.97	
year 2017	324	115.541	30.51	
year 2018	320	109.107	34.12	
year 2019	370	103.327	29.39	
year 2020	315	98.641	28.63	
TMM^{120} by parity				
1st lactation	520	107.747	30.59	**0.00000**
2nd lactation	475	106.615	28.02	
3rd lactation	388	105.017	31.69	
4th lactation	296	103.129	28.67	
5th+ lactation	331	90.176	29.47	

$^{**}P \leq 0.001$

The results thus obtained confirm the thesis expressed by us and other authors that the phenotypic variations of milk productivity and the manifestation of the genetic potential of sheep are directly related to the year of production, which largely takes into account the influence of so-called external (non-genetic) factors. Most of them (production system, rearing conditions, health status, complete nutrition according to the physiological condition of the animals, availability of pastures and nutritional composition, management and marketing strategy in the farm, the specific daily care of the animals, etc.) can be controlled and systematically improved.

Table 3. Overall mean and analysis of variance for a milk yield per standard 120-day milking period (consecutive lactation) by production year (l)

Variable	**n**	***x***	**SD**	**P-value**
1st lactation				
year 2015	117	97.887	26.19	**0.000022**
year 2016	84	101.530	30.32	
year 2017	64	114.851	28.81	
year 2018	84	115.413	34.56	
year 2019	94	114.152	28.90	
year 2020	77	107.427	31.19	
2nd lactation				
year 2015	92	98.501	21.69	**0.000003**
year 2016	83	100.319	27.418	
year 2017	79	118.545	29.53	
year 2018	57	115.306	30.03	
year 2019	87	107.511	28.65	
year 2020	77	103.408	26.34	
3rd lactation				
year 2015	77	90.188	28.07	**0.000000**
year 2016	75	99.111	26.89	
year 2017	64	132.263	30.71	
year 2018	59	107.459	36.63	
year 2019	51	108.254	24.02	
year 2020	62	97.469	24.86	
4th lactation				
year 2015	35	94.052	23.11	**0.000001**
year 2016	55	96.398	31.09	
year 2017	62	105.905	24.63	
year 2018	54	122.330	25.70	
year 2019	52	98.507	28.01	
year 2020	38	95.744	20.05	
5th+ lactation				
year 2015	26	81.301	25.28	**0.000544**
year 2016	37	99.572	31.09	
year 2017	55	103.431	30.87	
year 2018	66	86.384	30.29	
year 2019	86	87.253	27.55	
year 2020	61	84.532	26.33	

$^{*}P \leq 0.001$

CONCLUSIONS

The total milk yield of the sheep for a 120-day standard milking period (103.379 l ±30.34) is close to the minimum threshold limit for the Elite class of the population. The same, it is within 94.798- 115.541 l, during the individual production years and is the highest in the sheep of the 1st lactation (107.747 l).

A significant effect of the production year factor on the total milk productivity for a 120-day standard milking period and the milk yield of ewes per consecutive lactation were established.

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EFFECT OF SOME FACTORS ON THE BIOLOGICAL PROLIFICACY OF SHEEP FROM THE NORTH-EAST BULGARIAN MERINO BREED

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Abstract

The subject of the study were 617 sheep from the Northeast Bulgarian Fine-Fleece Sheep Breed (NBFF) - Shumen inter-breed type, born from 2013 to 2018 and bred in the Scientific Center for Agriculture - Targovishte. The biological prolificacy trait at different ages has been investigated. There were 1839 observations of biological prolificacy from the first to the fifth lambing. The influence of the factors - breeding line, type of mating and year of birth was researched. The variance was analyzed on the basis of a multifactorial linear statistical model for each study age (consecutive lambing). The linear affiliation has a significant effect on biological prolificacy up to the third lambing. The type of mating has a significant effect on biological prolificacy at first lambing only. Sheep from the line No. 61 with FecB gene from Booroola Merino are superior in prolificacy the purebred and the lines with Australian Merino genes up to 4.5 years. The biological prolificacy increaseed by the second lambing and was highest at 3.5 years (117%).

Key words: *breeding line, Northeast Bulgarian Fine-Fleece Breed (NEFF), prolificacy, sheep, type of mating, year of birth.*

INTRODUCTION

Biological prolificacy largely determines the profitability of production in the modern sheep farm. Meat is one of the main products, forming 50-60% of the total income in the dairy sector in our country (Stancheva & Staykova, 2009), from 52.13% to 69.34% in the aboriginal breeds (Staykova, 2005) and over 70% in the meat and fine fleece sheep farming (Boykovski et al., 2006). Our authors investigated the heritable variance of prolificacy in sheep and proved that it is low and the expected efficiency of mass selection on this trait is insignificant (Boykovski et al., 2002; 2009; Slavov et al., 2008; Stancheva et al., 2005; Stancheva, 2013). Through genetic improvement, good results have been obtained in a much shorter time. The Australian Booroola Merino (BoM) is a valuable genetic resource for increasing biological prolificacy in fine fleece and other sheep breeds worldwide. The effect of the introduction of the F gene (FecB) from the Booroola Merino breed for high prolificacy in sheep has been studied by a number of authors over a period of about 40 years (Turner, 1978; Bindon, 1984; Davis & Hinch, 1987; Davis et al., 1998; Fogarty, 2009; Abraham & Thomas, 2012). Tsonev (2014), Boykovski et al. (2018) and Slavova (2019) comment on the levels of the main productive traits in Bulgarian fine fleece breeds and their crosses with Booroola Merino.

Frozen seminal fluid from two Booroola Merino rams was imported into Bulgaria in 1988, evaluated for progeny at the Haldon station - New Zealand. As a result of introduction of the F-gene from Booroola Merino into the genetic structure of the flock of North-Eastern Bulgarian Fine Fleece Breed (NBFF) sheep at Scientific Centre for Agriculture - Targovishte, two breeding lines were formed, originating from the homozygous for this gene rams No. 61 and No. 377, of which only the first is currently active. Slavov et al. (2008) found an increase in genetic variance when including genetic components from the Booroola Merino, Australian Merino and Ile de France breeds in the NBFF-Dobrudzhan type population. Laleva et al. (2014) published positive results of a comparative study of ovulation rate, prolificacy and live weight at birth traits in Thracian fine fleece ewes and their crosses with Booroola Merino.

The evaluation of the variance in the intra-breed linear structures, carriers of the F gene (FecB) in the only nucleus flock of sheep from the North-Eastern Bulgarian Fine Fleece breed - Shumen type (reared in Scientific Centre for Agriculture - Targovishte) is a necessary condition for choosing appropriate selection methods and schemes for extending genetic progress in the trait prolificacy throughout the population. The assessment of the influence of other genetic and environmental factors complements the information on which scientifically based decisions in the selection process are based. This motivates the present development.

The purpose of the study is to determine the influence of some factors on the biological prolificacy of sheep from the North-Eastern Bulgarian Fine Fleece Breed (NBFF) - Shumen type.

MATERIALS AND METHODS

The subject of the study are 617 sheep from the North-Eastern Bulgarian Fine Fleece breed (NBFF) - Shumen type, born in the period from 2013 to 2018 and raised in the Scientific Center for Agriculture in Targovishte. The biological prolificacy trait at different ages was investigated. 1839 observations of the biological prolificacy trait were recorded from the first to the fifth lambing of the ewes. The influence of the factors - breeding line, type of mating and year of birth was researched. An analysis of variance was performed based on a multivariate linear statistical model for each studied age (consecutive lambing), which has the following form:

$$\mathbf{Y}_{ijko} = \mu + \mathbf{A}_{i\,(1-6)} + \mathbf{B}_{j\,(1-2)} + \mathbf{C}_{k\,(1-6)} + \mathbf{e}_{ijko}$$

where:

μ - total mean;

$\mathbf{A}_{i\,(1-6)}$ - effect of the breeding line factor (fixed) - 6 levels (lines);

$\mathbf{B}_{j\,(1-2)}$ - effect of the type of mating factor (fixed) - 2 levels (intralinear and interlinear);

$\mathbf{C}_{k\,(1-6)}$ - effect of the factor year of birth (fixed) - 6 levels (2013-2018)

$\mathbf{e}_{ijk}$ - residual effects, ≈ N (O, δe2)

The differences between the levels of the studied factors were established on the basis of the degree of distribution measured by Student (Hayter, A. 1984):

$(yi - yj)/S \sqrt{(1/ni + 1/nj)/2}$

where: (yi - yj) - differences, between the average values of the levels of the studied factor; S - square deviation; ni and nj - number of observations (individuals) for the respective levels.

RESULTS AND DISCUSSIONS

The linear affiliation (Table 1) has a significant influence on the phenotypic manifestation of the trait biological prolificacy ($P<0.001$, $P<0.01$, $P<0.05$) up to 4.5 years. The value of the F-criterion is the highest at 3.5 years ($F = 10.794$) ($P<0.001$) when animals reach full body growth and development, as well as the most complete reproductive performance in our study. The factor - type of mating had a significant influence on the prolificacy of the first lambing only ($F = 4.175$) ($P<0.05$). According to the results obtained in our study, the year of birth does not have a reliable influence on the phenotypic manifestation of the biological prolificacy trait. The coefficients of variation of trait studies are high and range from 24.03% to 30.40%, which is characteristic of the trait and similar to data published by other authors. The variance of the first lambing in our study is the smallest, and then it increases until the third and gradually decreases until the last studied age. Stancheva et al. (2020) found an average coefficient of variation CV = 29% for the biological prolificacy of the same herd in which we conducted our study. The analysis of the results of the study shows the dominant influence of the genetic factor - linear affiliation on the trait of biological prolificacy. Staykova et al. (2022) found that the linear affiliation did not have a significant effect on wool yield, clean wool yield and pure fiber traits in sheep from the North-East Bulgarian Fine Fleece Breed (NBFF) - Shumen type.

Table 1. Analysis of variance of the trait biological prolificacy

Sources of variance	df	F	P	CV%
2.5 years of age				
Breeding line	5	7.049	***	25.49
Type of mating	1	4.175	*	
Year of birth	5	0.099	n. s.	
3.5 years of age				
Breeding line	5	10.794	***	28.99
Type of mating	1	0.137	n. s.	
Year of birth	4	0.324	n. s.	
4.5 years of age				
Breeding line	5	3.368	**	30.40
Type of mating	1	0.470	n. s.	
Year of birth	3	0.179	n. s.	
5.5 years of age				
Breeding line	5	0.548	n. s.	29.63
Type of mating	1	0.421	n. s.	
Year of birth	2	0.699	n. s.	
6.5 years of age				
Breeding line	5	0.736	n. s.	24.03
Type of mating	1	0.244	n. s.	
Year of birth	1	0.048	n. s.	

*** - P< 0.001; ** - P< 0.01; * - P< 0.05

The results in Table 2 show that ewes from line No. 61, descendants of a purebred Booroola Merino ram and homozygous for the F (FecB) gene, presented with positive LS-scores for the trait prolificacy up to 5.5 years (P<0.001, P<0.01, P<0.05), at 6.5 years of age the scores are close to the LS mean. Dimitrov (1997) published data on 88% superiority of the half-breed crosses compared to the purebred starting forms. Boykovski et al. (2002) found similar results for the same breed - a real increase of 86.80%, with a theoretically expected 82.50%. Slavova (2019) reported a relatively low live weight of ewes from line No. 377 with Booroola Merino genes in the Thracian Fine Fleece Breed, but with higher biological prolificacy than their peers. The purebred NBFF line No. 239 in our study was characterized by negative LS-estimates for the trait prolificacy at all ages studied, and line No. 583 - up to 4.5 years, after which the mean for the line was close to the total for the sample. The Australian Merino bloodlines in our study - No. 755, No. 777 and No. 845 showed negative LS-estimates for biological prolificacy up to 4.5 years of age. At the fourth and fifth lambing, the LS-estimates were divergent, close to the LS mean and without statistical assurance of the differences. After a long breeding activity, the linear contrasts, when compared with the purebred animals, become narrower, but they are indicative, especially at 3.5 and 4.5 years. The total LS average showed the highest level of trait prolificacy at 3.5 years (117.30%). Slavov (2007) studied the productivity of the North-East Bulgarian Fine Fleece Breed (NBFF) - Dobrudzhan type and indicated 118.60% and 130.10% biological prolificacy, respectively, at the first and second lambing. Stancheva et al. (2020) found a lower prolificacy (110.40%) at 3.5 years when studying the same herd of the Shumen type, and the authors recorded the highest prolificacy at the fourth lambing (115.80%). The data from Table 2 in our study confirm the positive effect of the introduction of the F (FecB) gene for high prolificacy in the genetic structures of the North-Eastern Bulgarian Fine Fleece breed.

The results in Table 3 reflect the effect of the type of mating factor. At 2.5 years old, the sheep, a product of inter-line mating, are presented with a positive deviation from the average for the studied sample. At later ages, LS-estimates are divergent, close to the mean and without statistical assurance of differences.

Table 2. LS-estimates (LSE) of the effect of breeding line on biological prolificacy (lambs/ewe) by parity

Line Factor levels	Age, years (parity)														
	2.5			3.5			4.5			5.5			6.5		
	n	LSE	LSM ± SE	n	LSE	LSM ± SE	n	LSE	LSM ± SE	n	LSE	LSM ± SE	n	LSE	LSM ± SE
№ 61	51	0.195 ABabc	1.299± 0.040	42	0.326 ABCDa	1.499± 0.052	33	0.201 alm	1.367± 0.062	13	0.077	1.230± 0.092	5	-0.049	1.001± 0.115
№ 239	126	-0.065 A	1.039± 0.025	106	-0.088 A	1.085± 0.033	77	-0.089 a	1.076± 0.040	44	-0.046	1.107± 0.052	10	-0.061	0.991± 0.084
№ 583	137	-0.056 B	1.047± 0.024	116	-0.061 B	1.112± 0.031	85	-0.060 l	1.105± 0.038	54	0.005	1.158± 0.047	21	0.001	1.053± 0.057
№ 755	144	-0.015 a	1.089± 0.024	140	-0.060 C	1.113± 0.029	89	-0.012m	1.153± 0.038	56	-0.054	1.099± 0.046	20	0.045	1.097± 0.058
№ 777	110	-0.027 b	1.077± 0.027	98	-0.072 D	1.101± 0.034	71	-0.022	1.144± 0.043	33	-0.020	1.133± 0.059	12	0.113	1.164± 0.077
№ 845	49	-0.032 c	1.072± 0.040	40	-0.044 a	1.129± 0.053	33	-0.017	1.149± 0.061	17	0.038	1.191± 0.082	7	-0.048	1.003± 0.097
Overall LS-mean, μ	**617**	**1.104 ± 0.013**		**542**	**1.173 ± 0.017**		**388**	**1.166 ± 0.020**		**217**	**1.153 ± 0.028**		**75**	**1.052 ± 0.034**	

Significance of differences within columns – when symbols identical:
A to Z – P< 0.001; a to k – P< 0.01; l to z – P< 0.05

Table 3. LS-estimates (LSE) of the effect of type of mating on biological prolificacy (lambs/ewe) by parity

Type of mating Factor levels	Age, years (parity)														
	2.5			3.5			4.5			5.5			6.5		
	n	LSE	LSM ±SE	n	LSE	LSM ±SE	n	LSE	LSM ± SE	n	LSE	LSM ± SE	n	LSE	LSM ± SE
Interlinear	262	-0.025	1.079±0.019	233	0.006	1.179±0.025	167	-0.014	1.152±0.031	127	-0.015	1.138± 0.035	38	0.016	1.068±0.047
Between the lines	355	0.025	1.129±0.016	309	-0.006	1.167±0.021	221	0.014	1.179±0.026	90	0.015	1.168± 0.038	37	-0.016	1.036±0.047
Overall LS - mean, μ	**617**	**1.104 ± 0.013**		**542**	**1.173 ± 0.017**		**388**	**1.166 ± 0.020**		**217**	**1.153 ± 0.028**		**75**	**1.052 ± 0.034**	

The results in Table 4 show the influence of environmental effects on biological prolificacy in sheep born and produced in subsequent years. Animals born in 2014 presented with negative LS-scores until the third lambing, after which values were close to the sample mean. Those born in 2017 show a positive deviation from the average LS of the two ages studied. Year of birth as a factor has a relatively weak effect on prolificacy with no significant differences between groups. Staykova et al. (2022) found that the year of birth had a significant influence on the wool productivity when studying the same herd. The trait level results in our study confirm those from the analysis of variance in Table 1. Regardless of the differences present in the absence of statistical certainty, it confirms the fact that prolificacy is a relatively conservative trait and cannot be relied upon for better reproductive outcomes by optimizing feeding and rearing conditions. The methods of genetic improvement, by increasing the potential for biological prolificacy, make it possible to achieve better and faster results in practice.

Table 4. LS-estimates (LSE) of the effect of year of birth on biological prolificacy (lambs/ewe) by parity

Year of birth Factor levels	Age, years (parity)														
	2.5			3.5			4.5			5.5			6.5		
	n	LSE	LSM ± SE	n	LSE	LSM ± SE	n	LSE	LSM ± SE	n	LSE	LSM ± SE	n	LSE	LSM ± SE
2013	66	-0.008	1.096± 0.035	66	-0.007	1.166± 0.042	65	0.015	1.180± 0.045	61	0.029	1.182± 0.046	35	-0.007	1.045± 0.049
2014	81	-0.008	1.097± 0.032	77	-0.021	1.152± 0.038	74	-0.005	1.161± 0.042	41	0.004	1.157± 0.053	40	0.007	1.059± 0.044
2015	160	0.008	1.003± 0.023	160	-0.010	1.163± 0.028	132	0.011	1.177± 0.033	115	-0.033	1.120± 0.035			
2016	139	-0.005	1.099± 0.026	127	0.011	1.184± 0.032	117	-0.021	1.145± 0.036						
2017	111	0.013	1.117± 0.028	112	0.027	1.200± 0.033									
2018	60	-0.001	1.103± 0.036												
Overall LS-mean, μ	**617**	**1.104 ± 0.013**		**542**	**1.173 ± 0.017**		**388**	**1.166 ± 0.020**		**217**	**1.153 ± 0.028**		**75**	**1.052 ± 0.034**	

CONCLUSIONS

The linear affiliation of sheep from the North-Eastern Bulgarian Fine Fleece Breed (NBFF) - Shumen type has a significant effect on biological prolificacy up to the third lambing. The type of mating has a significant effect on biological prolificacy at first lambing only. No effect of year of birth on biological prolificacy was observed at any of the ages studied.

Sheep from the line No. 61 with FecB gene from Booroola Merino are superior in prolificacy the purebred and the lines with Australian Merino genes up to 4.5 years.

The biological prolificacy increaseed by the second lambing and was highest at 3.5 years (117%). Prolificacy increases at the second lambing and gradually decreases until 6.5 years.

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THE EFFECTS OF ALTERNARIOL MYCOTOXIN ON CELL CYCLE AND PROLIFERATION OF PORCINE BLOOD CELLS

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Abstract

Feed contaminants, especially mycotoxins are responsible for important economic loses in swine industry, pigs being very susceptible to contamination with mycotoxins. Alternariol (AOH) is a mycotoxin synthesised by fungi of genus Alternaria and is a common contaminant of different raw materials, as cereal and cereal products that represent important ingredients of swine diets. Recent literature data have shown that in human cell model, AOH can cause DNA damage and induce oxidative stress. However, the effect of AOH in swine it less documented. Consequently, the aim of our research was to investigate how AOH can affect the proliferation, apoptosis, death, and cell cycle using an in vitro model represented by PBMCs (peripheral blood mononuclear cells) isolated from pig`s blood. Cells were stimulated or not with phorbol 12-myristate13-acetate-ionomycin (PMAI), exposed for 4h to different concentrations of AOH (1-100 µg/mL). Our study shows that AOH was able to affect cell proliferation, apoptosis, cell death and cell cycle of porcine PBMCs, with negative effects on the animal health.

Key words*: Alternariol, apoptosis, cell cycle, oxidative stress, pigs.*

INTRODUCTION

Mycotoxin contamination of cereals, fruits, but also of other plant-based products remains a topical issue at the global level, especially in terms of the health of pigs, which mainly consume feed obtained by mixing these types of ingredients. Regarding sensitivity, several studies have shown that pigs are more sensitive than other species to the action of mycotoxins (Oswald et al., 2005). Pigs have also been utilized as models for human nutrition, including metabolic disorders, obesity, bariatric surgery, neurology, brain imaging, food allergies, and alcohol consumption, also they are a suitable model for humans (Roura et al., 2016).
Alternariol (AOH) is a secondary metabolite synthesized by *Alternaria alternata* fungi, generally contaminating fruits, but also cereal crops and vegetables (Tournas & Stack, 2001). It is known that AOH, like other mycotoxins produced by *Alternia* molds, have a high thermal stability, contamination can also occur at low temperatures during storage (Asam et al., 2009). Moreover, there are currently no guidelines regarding the concentrations that can be tolerated both in the case of humans and in the case of animals Alexander et al., 2011), although *in vitro* investigations have suggested that AOH can alter the DNA (Pfeiffer et al., 2007), and it can also lead to the appearance of some mutations (Brugger et al., 2006). The AOH mycotoxin can act as a topoisomerase II-a inhibitor (Fehr et al., 2009), this could serve as the primary mechanism by which AOH could break the DNA double strand. There are no data on the toxicity of AOH or other *Alternaria* toxins in other animals, including pigs. *In vitro* studies on RAW 264.7 macrophages have demonstrated that AOH can trigger the oxidative response through the generation of ROS (reactive oxygen species) in addition, AOH could be capable of producing oxidative damage at the level of DNA (Solhaug et al., 2012). Reduced cell proliferation was linked to this effect, which led to an accumulation of cells in the G2/M phase of the cell cycle (Lehmann et al., 2006). Although several studies indicate that AOH causes cell cycle arrest, a more accurate and thorough description of how AOH interferes with cell proliferation, viability, and death is required (Brugger et al., 2006; Wollenhaupt et al., 2008; Schreck et al., 2012).
Our study's objective was to use flow cytometry to assess the effects of AOH on ROS production, cell cycle, cell apoptosis and necrosis in swine

PBMCs (peripheral blood mononuclear cells) newly isolated from peripheral venous blood.

MATERIALS AND METHODS

Cell cultures

The PBMCs were separated using Li-heparin tubes (Vacutest Kima, Arzergrande, Italy), from freshly collected peripheral venous pig blood. Blood collection was carried out in compliance with the current rules and regulations (EU Council Directive 98/58/EC and Law 06/2004 for the care and safeguarding of animals utilized in experiments). The study was performed also following the rules of Ethics Committee of the National Research and Development Institute for Animal Nutrition and Biology, Balotești, Romania (Ethics Committee no. 41/2023). The blood was diluted 1/2 in sterile PBS and placed on Histopaque-1077 medium (Sigma Aldrich, St. Louis, Missouri, US) and centrifuged at 1500 rpm to separate the PBMC from the rest of the blood components. After separation, the white ring of lymphocytes was recovered, the red blood cells were lysed, and the isolated PBMCs were cultivated in culture plates with 24 wells, at a density of 5 x 10^5 cell/mL, in complete RPMI-1640 culture medium (100 µg/mL-streptomycin, 100 U/mL-penicillin, 0.025 µg/mL-amphotericin B, 4 mM-L-Glutamine and 5%-fetal bovine serum) (Sigma Aldrich, St. Louis, Missouri, US). The experiments were carried out for 4 hours in an environment enriched with 5% CO_2 at a temperature of 37°C.

Experimental design

To evaluate the effects of AOH on porcine PBMCs cells, two conditions were approached: unstimulated cells, and cells stimulated with para-methoxy-amphetamine (PMA) - 50 ng/mL and ionomycin (I) - 1 µg/mL. Stimulated cells were used as a model of inflammation as inflammatory process is often induced in pigs by various external stimuli such as bacteria, co-contamination with other mycotoxins, etc. AOH was diluted in dimethyl sulfoxide (DMSO), and in the case of both conditions with unstimulated or stimulated cells, the following concentrations of AOH were tested: 1, 5, 10, 50 or 100 µg/mL. When the incubation period ended, the effects of AOH on cell proliferation, apoptosis, necrosis, cell cycle, and oxidative stress were evaluated by flow cytometry.

Effects of AOH on Apoptosis

Through the use of the Muse Annexin & Dead Cell kit, the Muse Cell Analyzer system, and the Muse 1.5 Analysis software all provided by Merck, Darmstadt, Germany, the effects of AOH on PBMCs were assessed in the cases of the four stages of apoptosis (living, early apoptotic, live apoptotic, and death). In summary, flow cytometry analysis was performed after 100 µL of cell suspension (5 x 10^5 cells) was combined with 100 µL of reagent provided by the kit, and the mixture was incubated at room temperature in the dark for 20 minutes. A set of 2000 events was chosen for examination. The percentage of dead, living and apoptotic (early/late apoptosis) cells was the result of the apoptosis analysis; the graphs show the average of four separate tests.

Effects of AOH on Cell cycle

Cell cycle analysis was performed in compliance with the guidelines of Muse Cell Cycle kit (Merck, Darmstadt, Germany) provided by the manufacturer. For the purpose of analysis, 5 x 10^5 cells/mL were fixed in ethanol (70%) for 12 hours, diluted with the reagent from the kit, and then incubated in the dark for 30 minutes at room temperature. A set of 5000 events was chosen for examination. The cell distribution was reported as percentages (%) based on the cell cycle stages (G0/G1, S, or G2/M); the graphs show the average of four separate studies.

Effects of AOH on Oxidative Stress

The effects of AOH on PBMCs at oxidative level were evaluated using Muse Oxidative Stress Kit (Merck, Darmstadt, Germany). Oxidative stress at the level of PBMCs was determined by measuring reactive oxygen species (ROS). In summary, 190 µL of Oxidative Stress Reagent (Merck, Darmstadt, Germany) was combined with 10 µL of cells (1 x 10^5 cells/mL) and incubated for 30 minutes at 37°C. The results were expressed in percentages (%) of cell populations labelled ROS(+) or ROS(-).

Statistical analysis

The software used to assess the statistical significance of the changes between treatments was GraphPad Prism (9.3.0). One-way Anova and Fisher's exact test were performed. The outcomes were shown graphically, with p-values >0.1 indicating a trend and differences between the experimental treatments considered significant at a value of $p<0.05$. The statistical significance was graphically expressed as follows: ***-extremely significant [pϵ(0.0001, 0.001)], **-very significant [pϵ(0.001, 0.01)] and *-significant [pϵ(0.01, 0.05)].

RESULTS AND DISCUSSIONS

Effects of AOH on Apoptosis

Apoptosis is a normal cellular process, important in several biological systems. Inappropriate apoptosis can lead to the appearance of various pathologies such as neurodegenerative, autoimmune diseases or various forms of cancer (Cohen, 1997). It is known that mycotoxins can interfere with apoptotic processes, considering their spread in animal and human food, more studies are needed to identify the health risk to humans and livestock (Wang et al., 1996). Regarding the mycotoxins produced by *Alternaria* fungi, studies on secondary metabolites such as alternariol demonstrated the fact that these mycotoxins inhibit cell proliferation and induce apoptosis (Liu et al., 2007).

In our study, the effects of AOH on cell proliferation were evaluated both in the case of unstimulated PBMC cells and in the case of those stimulated with PMAI. As can be seen in Figure 1, AOH does not significantly influence the percentage of dead cells, but in the case of cell populations in late apoptosis, a significant increase is observed, directly proportional to the increase of AOH concentration. Moreover, once the AOH concentration increased, the population of live cells was significant reduced. It seems that in the case of unstimulated PBMCs, AOH can induce apoptosis, effects accentuated while the concentration of AOH increases, the percentage of total apoptotic cells being 10-30% higher at AOH concentrations above 10 μg/mL. Regarding the cells stimulated with PMAI, as can be seen in Figure 2, significant changes occur only in the case of exposure of stimulated cells to concentrations of AOH higher 50 μg/mL, respectively 100 μg/mL, where the percentage of dead cell populations increases significantly with 15.67% and 18.50%, while that of living cell populations decreases significantly with 14.77% and 18%. However, in the case of stimulated cells, AOH does not induce major modifications in the percentages of early/late/total apoptotic cells. Corroborating the data, AOH in high concentrations induced significant changes on the percentage of living or dead cells in the case of both studied conditions, PBMCs stimulated with PMAI or unstimulated.

Similar results were obtained by Solhaug et al., where the exposure of RAW 264.7 murine macrophages to AOH 60 μM for 24 and 48 hours reduce cell viability and induced the death of cells (Solhaug et al., 2012). Moreover, exposure to AOH was able to induce necrosis, apoptosis, and cell death in the case of Caco-2 human intestinal cells, where concentrations of AOH 15, 30, 60μM induced an increase in the percentage of apoptotic cells between 7.4-27.8% directly proportional to the increase in concentration (Fernández-Blanco et al., 2016). Other studies carried out on murine hepatoma cells (Hepa-1) showed that AOH 40 μM can induce a 3% upregulation in apoptotic cells after 48 hours of treatment (Schreck et al., 2012).

Mycotoxin co-contamination is a ubiquitous issue, and AOH has been shown to potentiate the effects of other mycotoxins. A study carried out on mouse blastocytes showed that even in low concentrations AOH (1 and 2 μM) potentiates the toxic effects of ochratoxin (OTA 8 μM) triggering cell apoptosis, in this case the main mechanism indicated that the accumulation of ROS (reactive oxygen species) is the main factor responsible for triggering the apoptotic process (Huang et al., 2021).

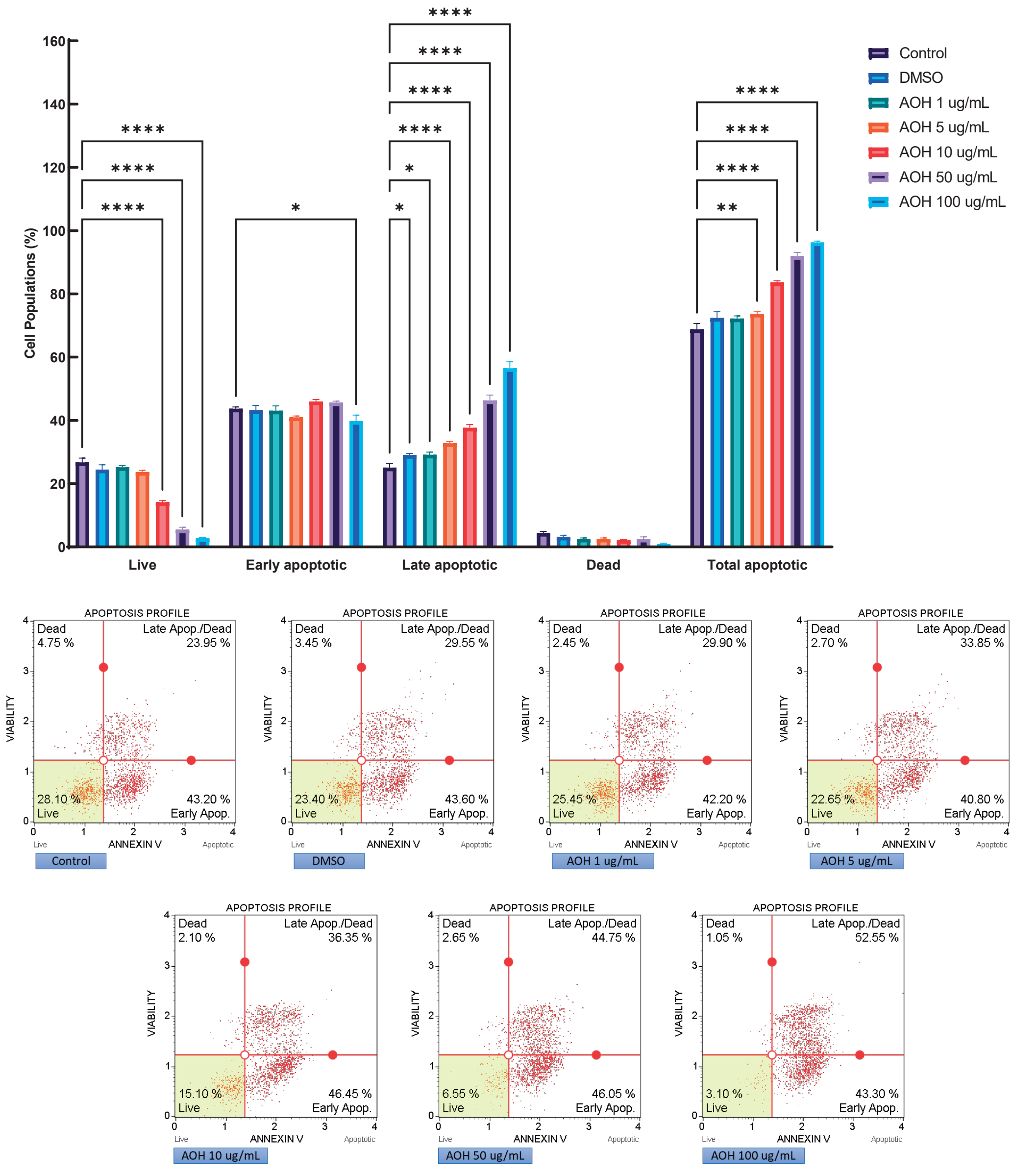

Figure 1. Effects of AOH on cell proliferation, apoptosis, and necrosis in unstimulated PBMCs

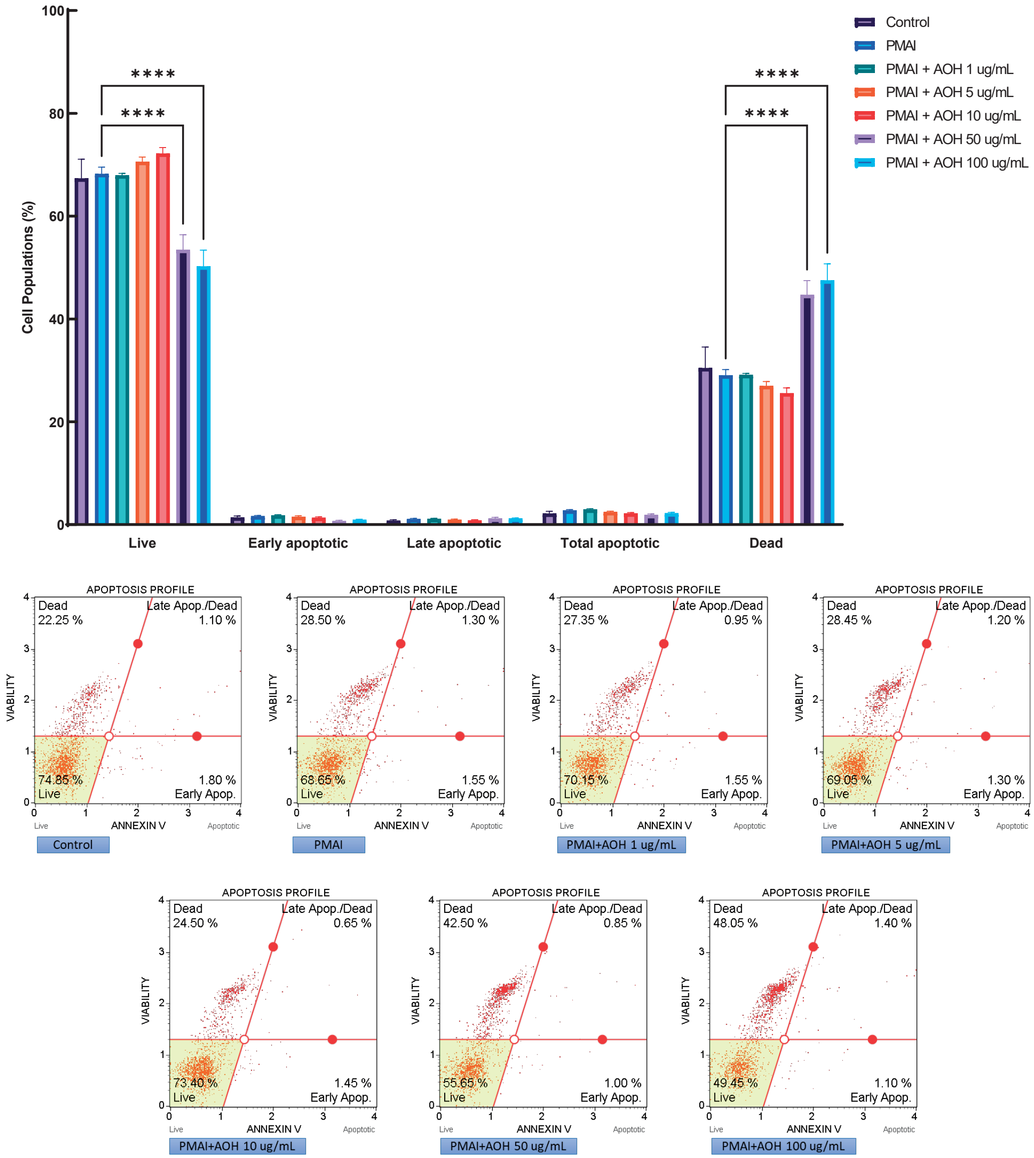

Figure 2. Effects of AOH on cell proliferation, apoptosis, and necrosis in PBMCs stimulated with PMAI

Effects of AOH on Cell cycle

The cell cycle is an intricate process, with three phases, which results in the duplication and transmission of genetic information (Schorl & Sedivy, 2007). In the first phase G0/G1, the cell responds to the stimulation of growth factors and extracellular mitogens, to then synthesize DNA in the S phase, and continue with mitosis (M), which has the final result of cell division (Israels & Israels, 2000). Any toxin or xenobiotic that has the ability to destroy DNA can alter the progress of the cell cycle, this could lead to neoplasia or various forms of cancer (Schafer, 1998). Regarding the changes in the cell cycle under the action of AOH, studies on RAW 264.7 mouse macrophages demonstrated that after 24 hours, a buildup of cell populations in phase G2/M may be caused by AOH 30 µM, also inducing morphological changes of the nucleus (Solhaug et al., 2012). Additional research revealed that AOH 60 µM significantly decreased cell populations in the phase G1/G0, while those in S and G2/M increased significantly after 48 hours of treatment (Fernández-Blanco et al., 2016). Regarding pigs, after exposure of porcine endometrial cells to AOH 12.5 µM for 24 hours, an arrest in the G0/G1 phase was observed, while the percentage of cells in phase S was reduced by more than 20% compared to the control (Wollenhaupt et al., 2008).

As demonstrated by Figure 3 for unstimulated PBMCs, the exposure to AOH doesn't induce any significant changes regardless of the concentration used. However, PMAI induces a significant decrease of cell populations (8.77%) in phase G0/G1, accompanied by a significant arrest in S phase (5.56%) (Figure 4). In stimulated cells, AOH induced a substantial increase of cell populations in phase G0/G1 in comparison with stimulated control (6.36% for 50 µM AOH) at the same time a notable decline in the quantity of phase S cells is observed for 50 µM AOH (8.33%) and 100 µM AOH (8.60%).

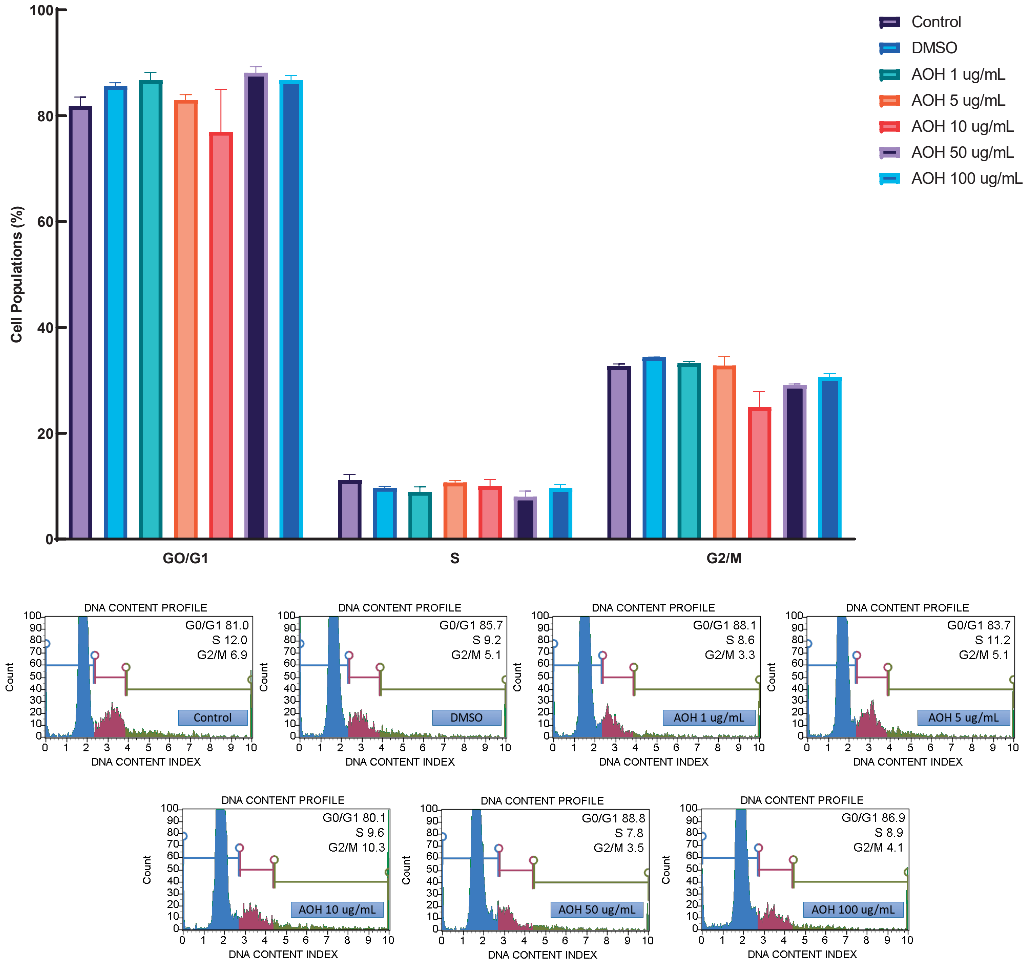

Figure 3. Effects of AOH on cell cycle in unstimulated PBMCs

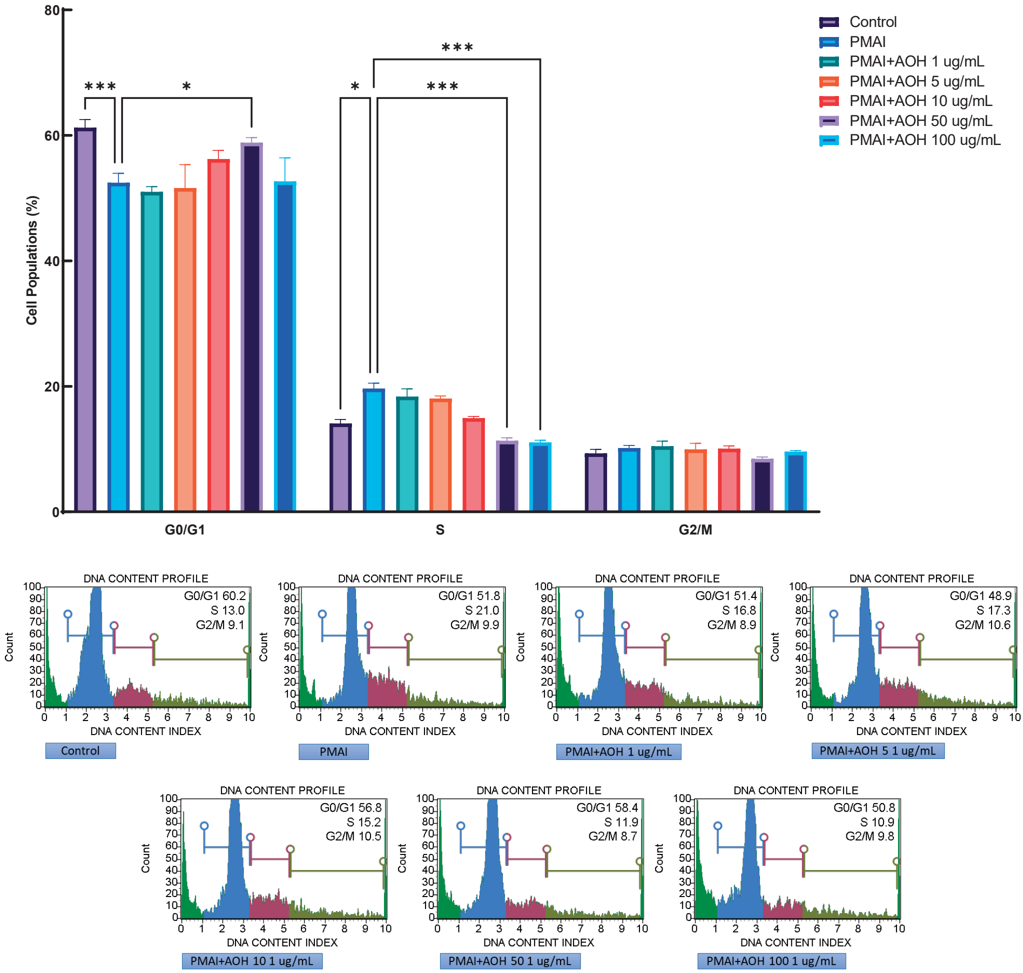

Figure 4. Effects of AOH on cell cycle in PBMCs stimulated with PMAI

Effects of AOH on Oxidative Stress

The role of oxidative stress is significant in many biological processes with important implications for the body, but it can also play a significant part in the etiology of several illnesses (Camhi et al., 1995). Most of the time, oxidative stress is caused by exposure to ROS (reactive oxygen species), such as the $HO^{\bullet}$ (hydroxyl radical), H_2O_2 (hydrogen peroxide) and $O_2^{\bullet -}$(superoxide anion) (Storz & Imlayt, 1999), causing damage to cell membranes, proteins, even nucleic acids (Crawford & Davies, 1994). Studies show that the oxidative response is closely related to apoptosis, the imbalance of redox processes at the cellular level being an important part of the signal transduction pathway(Buttke & Sandstrom, 1994), mediating apoptotic processes (Kannan & Jain, 2000).

Oxidative stress was evaluated under both conditions in unstimulated and PMAI stimulated PBMCs, as in the case of the cell cycle and apoptosis, by detecting changes in reactive oxygen species. For unstimulated cells, as can be seen in Figure 5, AOH was able to generate an increase in the percentage of ROS(+) cell populations in the case of higher concentrations of AOH, respectively 10 µg/mL (10.8%), 50 µg/mL (11.5%) and 100 µg/mL (16.3%). At the same time, a noticeable decrease of ROS(-) cell populations was observed in the case of the same concentrations 10 µg/mL (10.77%), 50 µg/ml (11,39%) and 100 µg/mL (16.49%). Moreover, similar outcomes were obtained in stimulated

PBMCs too. As can be seen in Figure 6, stimulation of cells with PMAI did not produce significant changes regarding the percentage of ROS(+)/ ROS(-) cells populations. However, the exposure to PMAI+AOH 50 μM/100 μM caused a rise in the percentage of ROS (+) cells of 23.66% and 21.77%, simultaneously with a significant decrease in ROS(-) 24.06% and 22.10%. This indicating that AOH can induce oxidative stress through the production of ROS. It seems that both in the case of stimulated cells and those stimulated with PMAI, at the level of oxidative stress, AOH is able to induce an increase in the level of ROS(+).

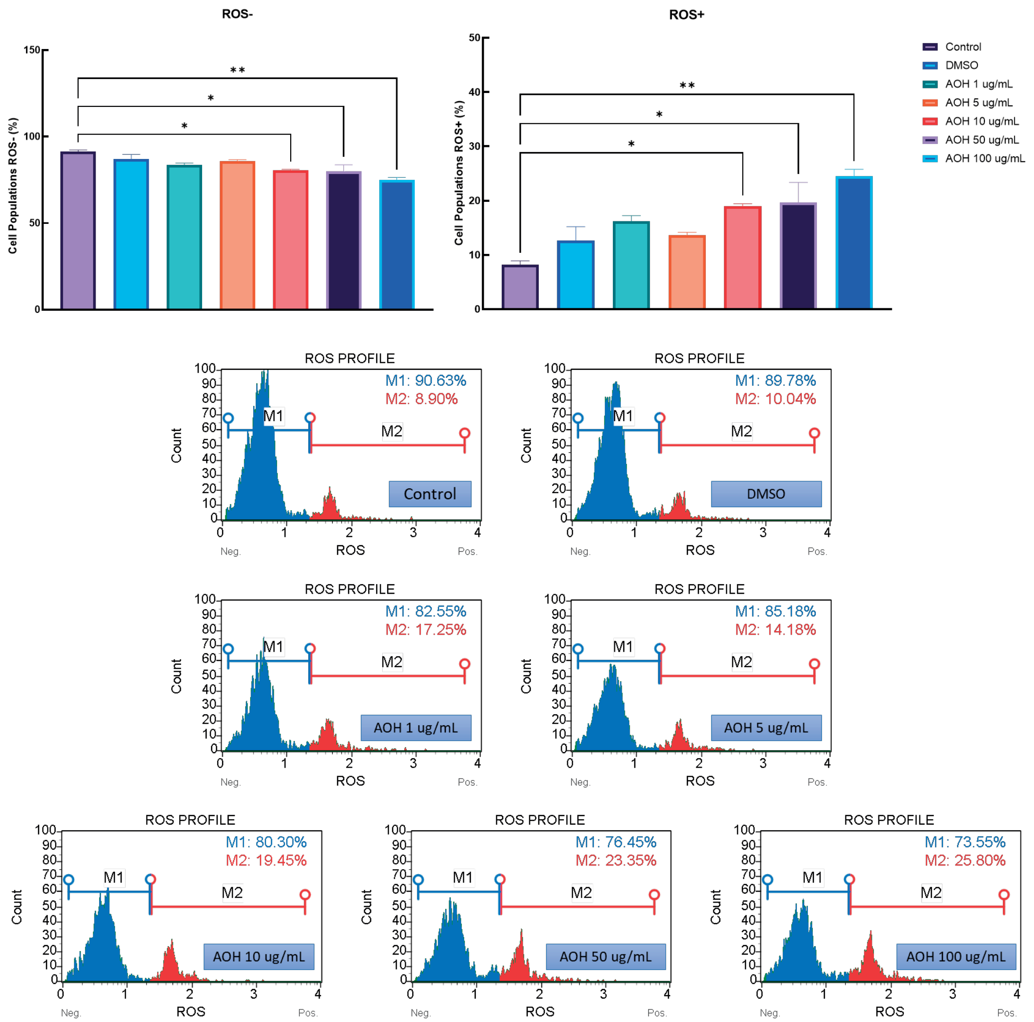

Figure 5. Effects of AOH on Reactive Oxygen Species in unstimulated PBMCs

It has been shown in several research that AOH can cause oxidative stress. In the case of the exposure of CaCO-2 cells to AOH (15, 30 and 60 μM), a reduction in the antioxidant enzymes' GSH, GPx and GR activity, along with DNA damage after was reported 24 hours of treatment (Fernández-Blanco et al., 2015). Additionally, it has been reported that AOH (5 mg/kg/body weight/day - 4 days) administered to pregnant mice was able to induce embryotoxicity and immunotoxicity by triggering apoptosis mediated by the accumulation of ROS (Huang et al., 2021).

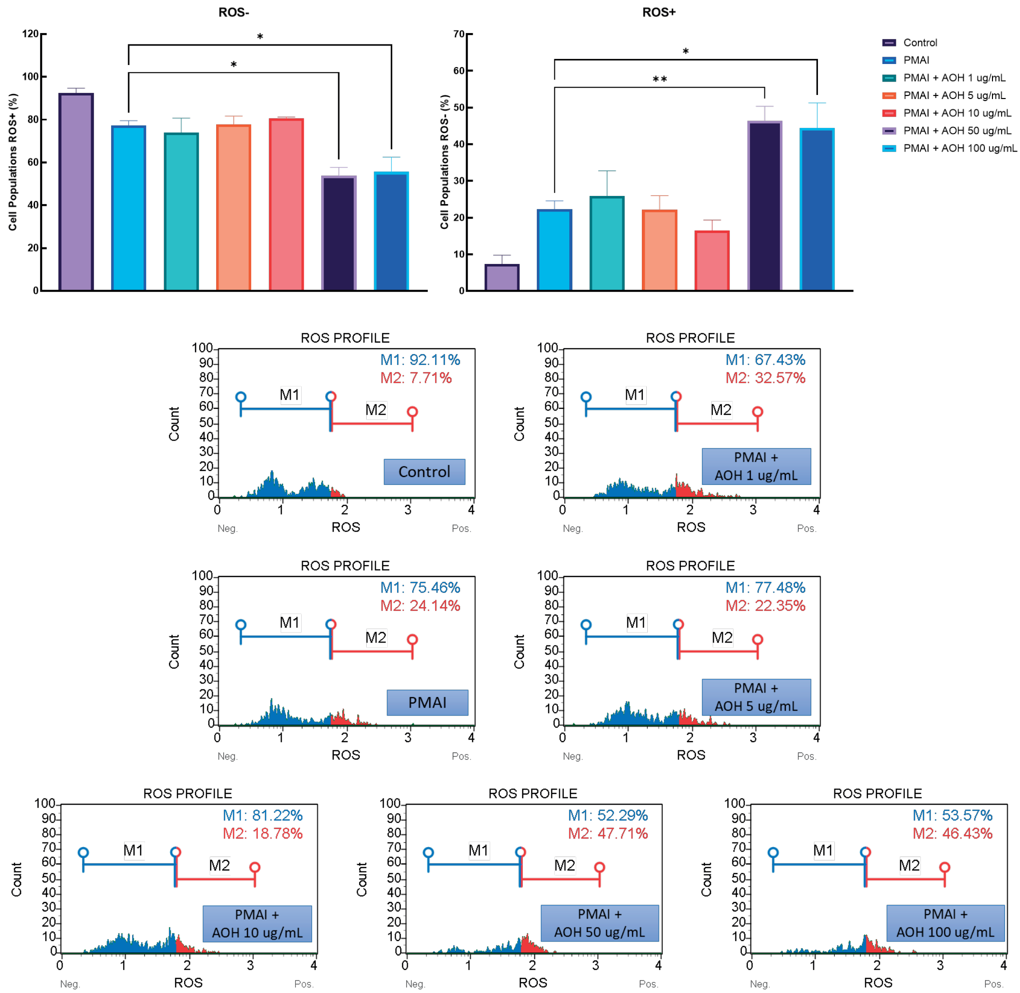

Figure 6. Effects of AOH on Reactive Oxygen Species in PBMCs stimulated with PMAI

CONCLUSIONS

Among animals used in agriculture, pigs are one of the most vulnerable species to mycotoxicosis. This happens mainly due to the diet rich in cereals, fruits, or other plant products, but also due to their innate sensitivity. Our study aimed to evaluate the effects of AOH (1-100 µg/mL) in PBMCs cells freshly isolated from peripheral venous blood from pigs The changes induced by AOH on apoptosis, cell cycle and oxidative stress were monitored under two conditions: unstimulated cells and cells stimulated with PMAI. Our results showed that AOH in high concentrations (50, 100 µg/mL) was able to increase cell death both in the case of stimulated cells and of stimulated ones. Moreover, regarding the unstimulated cells, the exposure to AOH resulted in an increase of the cells in late apoptotic stage for all the studied concentrations. Considering that data from the literature indicate that AOH can induce cycle arrest, the effects of AOH exposure on the cell cycle was also assessed. Thus, in the case of unstimulated cells, no significant changes produced by AOH were observed, but in the case of those stimulated with PMAI, it was observed that the addition of AOH 50 µg/mL and 100 µg/mL led to a rise in cell population counts in phase G0/G1, concurrently, a significant diminish of those in the phase S was also observed, compared to the stimulated control.

One of the mechanisms that could be responsible for the modulation of apoptotic processes is represented by the induction of oxidative stress through the accumulation of ROS. Our results, as in the case of those obtained in the case of apoptosis, shown that the proportion of ROS (+) cell populations could be significantly increased by AOH at 50/100 μg/mL, in the case of unstimulated cells, the increase of ROS(+) percentages starts even from AOH 10 μg/mL. This shows that the main mechanism of induction of apoptosis of AOH could be the production of ROS. Corroborating the obtained data, it can be concluded that in the case of porcine PBMCs cells, AOH was able to induce apoptosis and cell death, the main mechanism that could be responsible being through oxidative stress.

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INFLUENCE OF ENERGY BALANCE OF RATIONS ON MILK PRODUCTION IN COWS IN A PASTURE-BASED FEEDING SYSTEM

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Abstract

This study aimed to achieve the optimal energy balance of Holstein-Friesian cows, fed to produce 5,000-6,000 l of milk/cow per lactation (restricted production- Pr) on a pasture ration or 8,000-9,000 l of milk/cow per lactation (high production- Pm), on a more intensive feeding regimen using a partial mixed ration (pasture + concentrate). The mean of 4% fat-corrected milk (FCM) and standard deviation was 8,646 ± 1,162 l/cow per lactation for the Pm herd and 6,847 ± 787 l/cow per lactation for the Pr herd. In the first period of lactation, the balance degree estimated negative energy was lower in Pm cows than in Pr cows (-16.1 vs. -29.1 MJ/cow per day, respectively). As such, mobilization of body reserves was also lower in Pm cows, and this was reflected in lower concentrations of nonesterified fatty acids (0.7 vs. 0.8 mmol/l) and β-hydroxybutyrate (0.5 vs .0.7 mmol/l) and higher concentrations of glucose (3.5 vs. 3.3 mmol/l) and insulin for Pm and cows respectively, Pr.

Key words*: body reserve, fatt corrected milk, lactation, pasture, ration.*

INTRODUCTION

In cow milk production systems, based on pasture feeding, a system often practiced in Romania, it is necessary to choose a calving model, either compact in one season (seasonal calving) or in several seasons (fractional calving), in order to maintain a high level of pasture use and therefore to minimize feed costs (Mark et al., 2021). The ideal would be for farmers to aim to obtain a calf/year/female for reproduction.

However, a calving interval of 12 months is difficult to achieve due to the low fertility of the modern dairy cow Holstein-Friesian (HF), with productive potential for milk above average (Fulkerson et al., 2008).

As such, the fertility of the modern dairy cow has decreased as milk production has increased, particularly in the last 3 decades (Lucy, 2001) because the feeding potential has not kept pace with the potential increase in milk production. So, an increasing proportion of energy must come from the mobilization of body reserves, and this produces metabolic and endocrine changes that can affect fertility (Veerkamp et al., 2003). Mobilisation of body energy reserves is even greater, especially in grassland-based farming systems (Kolver & Muller, 1998). Various experiments were carried out for feeding dairy cows with high genetic potential for milk (producing 44 l/cow per day) with rations made up of unique feed mixtures, in short-term experiments - 4 weeks. Cows fed with these rations obtained 40 l milk/cow/day, and cows that had the ration provided only by high quality pasture, reached 30 l/cow/day (Garcia et al., 2007). The lower milk production of pasture-only cows was largely related to the lower net energy intake from ingested dry matter in that study and indicates, as also shown by Fulkerson et al., 2008, that cows with high genetic potential for milk production, cannot achieve this genetic potential of milk, only on pasture. The reasons for this are the higher energy expenditure in a pasture system, the lower intake capacity of cows when fully fed with a voluminous feed, and that cows are not able to maximize the use of pastures (Clark et al., 2005). Milk and meat animals fed with full-fat soy causes a high quality of milk, meat and fat (Bonea, 2021). Also, corn is considered an important source of protein for animal feed, the content varying between 8-12% (Bonea, 2020; Knight et al., 2004).

Transgenic crops have been widely adopted by growers and are a significant source of feed for livestock. Worldwide, a large part of the

forages used in animal feeds is transgenic (Bonciu, 2023a).
Animal feeds are frequent subject to contamination from diverse sources, including environmental pollution; therefore, the application of sustainable depollution strategies is needed (Bonciu, 2023b; Popa et al., 2016; Popa et al., 2022). A particularly negative impact on animal health is the contamination of feed with various chemicals. There are many methods of monitoring chemical contamination, but one very often used is the biological one, through the Allium test (Bonciu, 2023c).
Therefore, cows grazing at pasture must be supplemented with energy-dense concentrates in order to reach their genetic potential of milk and reduce the need to mobilize excessive amounts of body reserves at the beginning of lactation (Ferris et al., 2002; Dillon et al., 2003; Kennedy et al., 2003., Hennessy et al., 2020; Reist et al., 2003).
The decrease in dry matter intake near calving is only temporary (Bossaert et al., 2008). The cow's appetite will increase within a few weeks after calving, partly due to a decrease in insulin concentration. However, increasing the intake of dry matter does not correspond to the requirement of increasing energy. The peak of lactation occurs between the 4th and 8th week after calving, while the intake of dry matter only increases between the 8th and 22nd week after calving (Bossaert et al., 2008).
Cows with a significant negative energy balance (NEB) show a decrease in the immune system (Widmann et al., 2013; Le Blanc, 2008; Marin et al., 2020). Insufficient dry matter intake of DMI from meadows and calcium concentration around labour combined with negative NEB energy balance and loss of minerals or vitamins early in lactation result in a decreased immune system. This aspect can lead to serious health problems (Logue et al., 1999).
The most important thing is to be able to intervene on the energy level in time, so the cow will be helped to achieve optimal performance. When a cow has a good level of energy, it will contribute positively to milk production, fertility, immunity and calving (Cola & Cola, 2023). However, the ability of a more adequate diet to reduce the mobilization of the body reserves of dairy cows at the beginning of lactation and its subsequent effect on reproductive performance and health is equivocal.
Sutter & Beever (2000) accurately measured energy changes (by using calorimetric chambers and energy losses in feces and urine) during the first 7 weeks of lactation and found that approximately 95 kg of tissue was mobilized, while body weight (BW) loss was about 43 kg. Thus, although BW and BCS are practical indicators of energy status and should be measured, they do not accurately reflect the degree of mobilization of body reserves in early lactation (Buckley et al., 2000).
Interestingly, despite the fact that Rp cows had a greater degree and duration of estimated NEB and therefore a greater level of mobilization of body reserves, the times to onset of ovarian activity and overall reproductive performance were similar to of Hp cows.
In addition, high milk production itself has been associated with health problems, especially mastitis (Ingvartsen et al., 2003).
Therefore, the present study aimed to follow the feeding of Holstein Friesian (HF) dairy cows with average genetic potential for milk, on a system based on pasture rations, in order to obtain either a low milk production (about 6,000 l/cow per lactation) or a high milk production (about 9,000 l/cow/lactation), evaluating the effects on energy balance and metabolite changes.

MATERIALS AND METHODS

The experiment was conducted at SC Fenov SRL Dolj, during the years 2019-2020. The herd of cows of 180 heads was predominantly made up of Holstein-Friesian breed. From this herd, were formed 2 groups of milk cows, of 40 heads each, with approximately equal milk yields (5,600 l milk/previous lactation), of the same age, cows being at the 4^{th} lactation. Within each group, 40 cows were selected, depending on the date of calving (early autumn and winter), to be strictly monitored regarding the feed ration received.
Cows were fed on pasture and feed rations were supplemented with corn silage (*Zea mays*) up to 3 weeks before calving and with alfalfa hay (*Medicago sativa* L.), corn silage and

mixed concentrated feed per transition cow, in the last 3 weeks until calving, to meet their nutritional requirements. After calving, the cows were assigned to a restricted production group (Pr), in which the cows were fed sufficient rations that provided metabolizable energy (ME) and crude protein (CP) to produce approximately 6,000 l milk/lactation, and to another group, high production (Pm), in which the cows were fed to produce approximately 9,000 l/lactation (Table 1).

Table 1. The average composition of the ration and the analysis of the nutrients of cows according to the level of milk production

Ration composition (kg DM/day)	Nutrition system	
	Pr	Pm
Total dry matter (DM)	20.4 ± 3.4	24.9 ± 2.6
Pasture	8.2 ± 6.2	5.2 ± 4.3
Corn silage	3.7 ± 3.6	6.1 ± 1.8
Silage (alfalfa or sorghum)	1.5 ± 3.2	0.03 ± 0.3
Hay (alfalfa or oats)	2.7 ± 2.9	3.7 ± 1.4
Concentrate	3.7 ± 1.6	9.8 ± 1.7
Wheat	—	0.3 ± 0.7
Urea (g/day)	0.02 ± 0.03	0.02 ± 0.02
Calcium (g/day)	0.01 ± 0.01	0.02 ± 0.05
Nutrient analysis		
Digestibility DM (%)	69.3 ± 0.1	72.7 ± 7.2
CP (% of SU)	16.5 ± 0.1	17.5 ± 0.1
NDF (% of SU)	46.0 ± 0.1	37.4 ± 0.1
ADF (% of SU)	25.4 ± 0.1	20.5 ± 0.2
ME (MJ/kg LS)	9.8 ± 0.6	10.3 ± 0.3

(Pm) high production; (Pr) restricted production; (DM) dry matter; (CP) crude protein; (NDF) neutral detergent fibres; (ADF) acid detergent fibres; (ME) metabolisable energy; (LS) STAS (State Standard milk).

The animals of the Pr group received the entire amount of concentrate twice a day during milking, while the Pm cows received less than 4 kg/cow per day during milking, and the rest was mixed with the feed supplement as a partial mixed ration (RMP). The total ration offered was formulated to provide approximately 250 and 200 MJ of ME/cow/ day and a minimum of 17.5 and 16.5% CP, for Pm and Pr herds respectively.

The offered pastures were sown down with a complex mixture of grasses, *Dactylis glomerata*, over which was sown aristate ryegrass (*Lolium multiflorum* Lam.) in autumn and perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.). Pasture production available to both herds was limited due to drought during the experimental period; therefore, in some months (mainly from mid-May to July), stored fodder such as corn silage, alfalfa hay and oat hay (*Avena sativa* L.), were used for feeding, instead of pasture. Herds of cows started grazing according to the best practices of pasture use, according to the degree of leaf development, used as an indicator for marking the time of grazing. Pasture production was estimated before and after grazing using an automatic vehicle scale. The samples of herbs and mixed concentrate were taken every two weeks for the analysis of nutrients (DM, NDF, ADF, CP, ME, water-soluble carbohydrates and ash), as stipulated by the regulations in force, within the feed analysis laboratories of D.S.V.S.A. (Veterinary and Food Safety Department) Dolj and the Faculty of Agronomy of Craiova. Pasture samples were also taken to simulate grazing height.

Animal measurements and samples

Milk production was recorded twice a day using automatic flow meters (DeLaval, milking system). Milk samples were taken every two weeks at the morning and evening milking and were analysed for the determination of milk fat and protein and the number of NCS somatic cells, with a Lactoscan SCC.

Milk production was corrected to 4% fat content (LC 4%) to compare milk production between herds with different milk compositions. The body weight was recorded at weekly intervals, immediately after the morning milking, with an electronic cattle scale, and the physiological condition (scale 1 to 8) was visually recorded monthly by the same observer.

In the intensively monitored herd (n = 80 cows), blood samples were collected weekly, immediately after morning milking, from calving to 63 days postpartum, for analysis of urea, glucose and fatty acids. Samples with anticoagulant were placed on ice immediately after collection, while serum samples were kept at room temperature until the rennet formed. The samples were centrifuged to separate plasma and serum and were frozen at -20°C. The samples were subsequently analysed for glucose and urea concentrations in plasma by enzymatic colorimetry with a self-analyser within D.S.V.S.A. Dolj.

The composition of milk was measured at two weeks, during the first 9 weeks of lactation. As such, in odd weeks they were averaged to calculate the composition of milk for even weeks. Milk samples were taken from a quarter of each cow 3 times a week during milking, from calving to 120 days postpartum, divided into 4 aliquots with samples and frozen at -20ºC, until analysed.

The data obtained from both sets of data with 180 or 80 cows, was statistically processed, starting from the premise that the variance-covariance structure between times has a first-order autoregressive correlation. Before the analysis, the normal distribution of variables was evaluated and the data was transformed when necessary.

RESULTS AND DISCUSSIONS

Lactation performance (n = 180 cows)

The average lactation performance of Pr and Pm cows is shown in Table 2. Pm cows had milk production per lactation 38% higher than previous milk production per lactation, while Pr cows had milk production only 8% higher. Pm cows had a higher milk protein content (P = 0.04), but a lower milk fat content (P = 0.03) than Pr cows; therefore, milk fat and protein productions were 23 and 34% higher (P < 0.001) in Pm and Pr cows, respectively.

Table 2. Milk yield, components and content in fed cows (n = 180 cows)

	Nutrition system			P
	Pr	Pm	ESD	
Previous production of lactating milk (l/cow)	5,873	5,945	138	0.397
Milk production (l/cow per lactation)	6,353	8,181	248	<0.001
Yield LC4% (l/cow per lactation)	6,748	8,466	246	<0.001
Milk fat content (%)	4.39	4.23	0,01	0.037
Milk protein content (%)	3.11	3.20	0.05	0.042
Milk fat production (kg/cow per lactation)	286.4	354.1	11.6	<0.001
Milk protein yield (kg/cow per lactation)	202.1	270.9	6.7	<0.001

Pm - high production; Pr - restricted production; ESD - standard error of the difference; LC4% - fat-corrected milk production (4%).

Productive performance at the beginning of lactation (n = 78 cows)

At the beginning of lactation, milk production and milk composition were significantly different (P < 0.05) between herds (Figure 1). Milk production from Pm cows reached a peak of 34.9 l of LC4%/cow/day at about 7 weeks postpartum, while milk yield for Pr cows was 29.6 l of FCM/cow/day at about 4 weeks postpartum. Despite differences in milk production at the peak of lactation, the average daily yield of FCM from calving to 9 weeks postpartum was only about 4 l higher (P <0.001) in Pm cows (Figure 2).

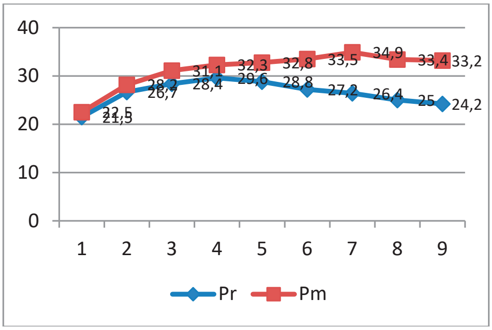

Figure 1. Milk production (l/day)

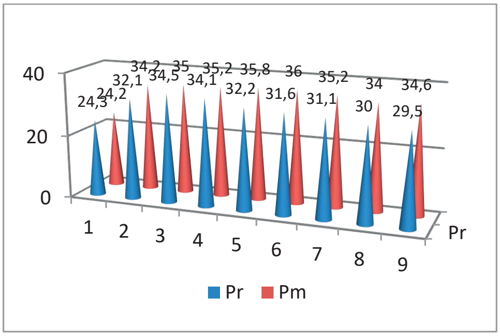

Figure 2. Yield LC4% (l/cow/day)

The protein content of milk was similar (P > 0.05) between herds, peaking at 5 weeks of lactation and then slowly increasing to 9 weeks of lactation (Figure 3). Milk fat content decreased in both herds up to 9 weeks of lactation, but the rate of decline was slightly faster in Hp cows than in Rp cows (Figure 4). Both milk protein yield and milk fat yield were higher (P < 0.05) in Pm cows than in Pr cows from week 4 to 9 postpartum.

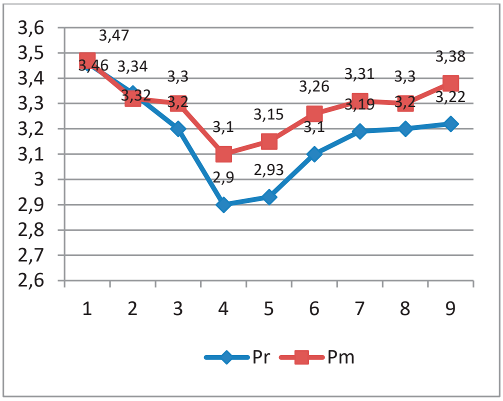

Figure 3. Milk protein content (%)

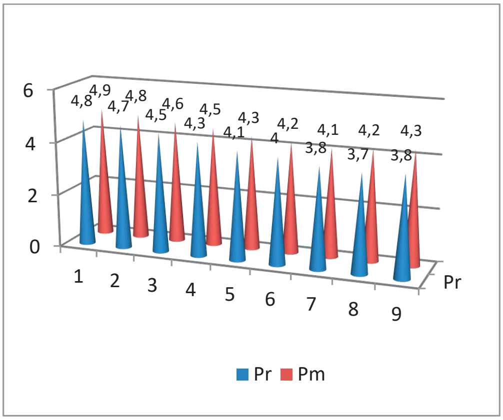

Figure 4. Milk fat content (%)

Energy balance and metabolic changes (n = 78 cows)

Body weigh (BW) and BCS (body condition scoring) decreased similarly at the beginning of lactation in both herds ($P > 0.05$). Pm cows lost 0.56 BCS units and 17.9 kg weight, and Pr cows lost 0.67 BCS units and 23.9 kg weight, from calving to 9 weeks postpartum. The estimated average energy balance during the first 9 weeks postpartum was significantly less negative ($P < 0.001$) in Pm cows than in Pr cows (Figure 5); estimated trend of negative energy balance (BEN) was -28 MJ/cow at 2 weeks postpartum in Hp cows, compared to -47 MJ/cow at 3 weeks postpartum in Rp cows. Changes in metabolites in circulation in cows at the beginning of lactation and their interactions with the feeding system and the postpartum week are shown in Figure 6.

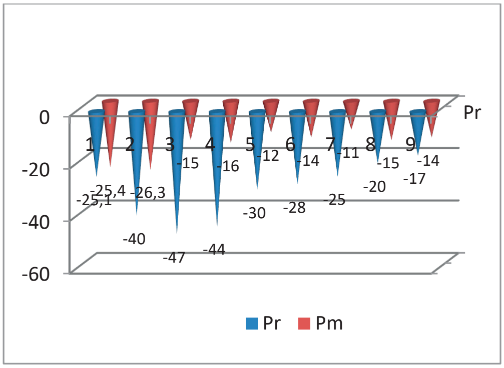

Figure 5. Energy balance (MJ/cow/day) in early lactating cows fed to achieve low (Pr) or high (Pm) milk

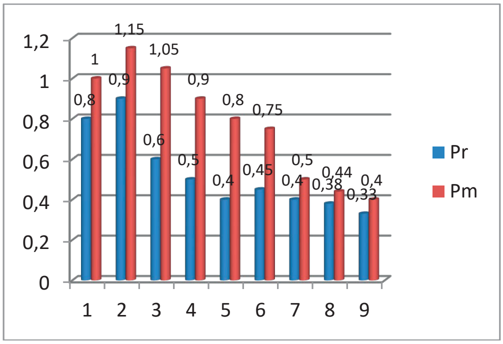

Figure 6. Concentrations of unsaturated fatty acids (mmol/l)

Changes in the concentration of non-esterified fatty acids indicated that both herds of cows had to rely on the mobilization of body reserves to meet the demand for nutrients for milk production, especially in the first 2 weeks postpartum. However, non-esterified fatty acid NEFA and β - hydroxybutyrate (BHBA) concentrations were lower ($P < 0.01$) in Pm cows than in Pr cows, reflecting a lower degree of fat reserve mobilization in the first ones (Figure 7). The glucose concentration was within the normal range in both herds, but was significantly higher ($P < 0.05$) in Pm cows compared to Pr cows between 3 and 5 weeks postpartum. In Pm cows, the concentration of IGF-I increased from calving to 9 weeks postpartum and was always significantly higher ($P < 0.001$) than in Pr cows at week 2 postpartum. Rp cows had an almost constant concentration ($P > 0.05$) of IGF-I for the same period (Figure 8).

The average urea concentration was within the normal range, but was significantly higher ($P < 0.001$) in Pm cows than in Pr cows (7.2 and 5 mmol/l, respectively), as expected from the composition of the diet. The effect of genetic merit was not significant ($P > 0.05$) for any of the metabolites measured. The calving season did not affect the concentrations of the metabolites measured, except that the concentration of IGF-I was higher ($P < 0.001$) in cows calving in early autumn than in cows calving in winter (80.2 vs. 58.7 ng/ml, respectively).

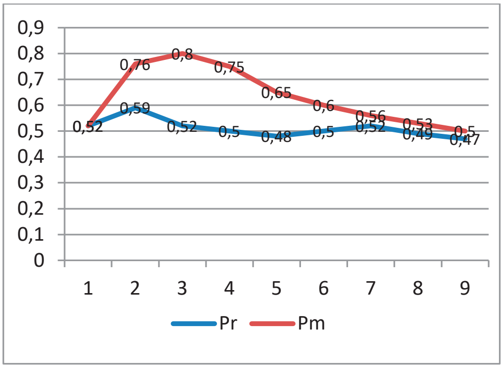

Figure 7. β-hydroxybutyrate (mmol/l)

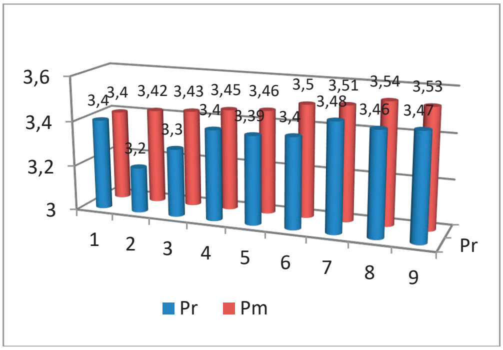

Figure 8. Glucose (nmol/l)

This study demonstrates that energy balance could be improved and fluctuations in key metabolites could be reduced when HF cows of average genetic merit were fed to increase milk production from a level of 5,945 l/cow per lactation when fed in a typical pasture system. In the pasture plus concentrates during milking variant, the production increases to 8,466 l of LC 4%/cow per lactation (SD = 1,162 l) when the cows received concentrated feed. The 30% increase in milk production did not affect reproductive performance, but the incidence of mastitis was increased. However, this characteristic must be followed by strict experiments on breeding indices in cows performing for milk production.

CONCLUSIONS

Milk production per cow in cattle with average production, fed on a typical pasture-based system, was improved by 30% during lactation when the energy intake was increased by offering a mixture of concentrated feed, and this led to an improvement in the estimated energy balance and a reduction in the mobilization of body reserves at the beginning of lactation. This achievement was without effect on the reproductive performance and health of cows, except for an increase in the incidence of mastitis that was associated with milking management.

Studies have found that HF cows, producing an average of 27.6 l/cow/day at first insemination, had lower reproductive performance than other lower milk producing breeds (25.6, 24.3 and French Normande, respectively).

These studies indicate a negative relationship between reproductive performance and milk yield, and this relationship appears to be true regardless of whether high milk yield has been achieved through improved management or genetic gain. However, the most likely reason for reduced fertility appears to be the greater need for high-producing cows to rely on body reserves to meet higher energy demands, which exacerbates changes in metabolic and hormonal factors during early lactation

He also found no effect on fertility when multiparous cows with high genetic merit were fed a grass-silage diet (winter feed) supplemented with concentrates in early lactation. Increasing concentrates (up to 14 kg/cow/day) in the diet reduced body condition loss and decreased stretch and NEB grade compared to cows supplemented with 5.5 kg/cow/day of concentrates.

It could be because the Rp cows in our study were less energy restricted. Although it was not possible to calculate the absolute level of energy restriction in these studies, our Rp cows received a level of concentrate similar to the highest level of concentrate fed in the other 2 studies. On average, at the start of lactation, Rp cows were fed approximately 90% of their energy requirement (estimated using actual milk production and energy provided in the diet), while Hp cows were fed 100% of their energy requirement of energy in relation to milk and the production achieved; therefore, differences in voluntary intake predominantly affected the level of energy intake of cows. This explanation is supported by the results who also found that when applied 5, 7 or 10 kg of concentrates/cow/day were fed to cows on pasture, reproductive performance did not differ between groups.

It was found that glucose concentration in early lactating cows increased rapidly if the cows were fed a high-energy diet. Despite the fact that the energy balance indicators used revealed significant differences in the mobilization of body reserves between herds, the changes in BW and BCS in Pm and Pr cows were similar. A higher level of energy supplementation (cows supplemented with 5.5 kg/cow/day rather than 1.4 kg/cow/day of concentrates) fed was also found to reduce the mobilization of body reserves and did not affect fertility. In that study, high genetic merit cows produced 8,707 or 6,014 l/cow per lactation, respectively, when fed high or low levels of concentrate, and these milk yields were similar to milk yield levels. from our study.

The performance of high and medium genetic merit HF cows at 3 different stocking rates and concentrate supplementation levels similar to ours (1.6 or 3.2 kg/cow per day) was also compared. They found reduced mobilization of body reserves at the higher level of supplementation, but found no difference in changes in BW and BCS during the first 20 weeks of lactation. The reason could be that in early lactation, changes in BW and BCS are poor indicators of energy balance.

Therefore, to maintain good reproductive performance, it is important to provide a diet that meets the cow's energy requirements, but additional energy is unlikely to improve fertility.

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THE USE OF PUMPKIN SEED CAKE IN THE DIETS OF FATTENING PIGS

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Abstract

The work presents the results of a study on the chemical composition of pumpkin seed cake with shell and its potential use in the feeding of fattening piglets. It was established that including pumpkin seed cake in the diets of meat hybrids at proportions of 4%/t and 7%/t of combined fodder did not have a negative impact on health, productive performance, carcass quality, and economic indicators. Tests showed that substituting soybean meal with pumpkin seed cake at a rate of 4%/t in the first fattening period and 7%/t of combined fodder in the second period provides an average daily growth increase of 789g, with a slaughter yield (hot carcass weight) of 80.53%, average backfat thickness at the 6/7 thoracic vertebra of 29.33 mm, 23.00 mm at the spine, 18.67 mm at the rump, eye muscle area of 42.69 cm², ham weight of 11.763 kg, and fat content in the Longissimus dorsi muscle of 4.53%, provided an economic benefit of 10.62 euros for each raised and fattened head.

Key words*: carcass; nutritional value; pumpkin seed cake; productive indices; slaughter yield.*

INTRODUCTION

Pigs, depending on their age, sex, physiological condition, etc., require assimilable energy, a specific level of protein, minerals, and vitamins, all in proportion to achieve efficient utilization of their genetic potential. To ensure high productivity, pig rations need to be controlled separately for approximately 32 nutritional elements, utilizing around 500 types of feeds and nutritional additives (Danilov & Donica, 2020). The use of concentrated feeds in pig nutrition, as well as their substitution with other feeds, is determined by the physiological requirements of different pig categories and a series of technical and economic conditions, such as: the ability to consistently provide quality feeds, their cost, the goal and the performances that are being pursued (Dinu et al., 1993; Coshman et al., 2023).

Industrial waste contains a complex range of nutritional compounds that, despite being of interest for animal feed, are not fully utilized and, on the contrary, are used irrationally and incompletely. Currently, the complex processing of raw materials and the rational management of waste are important issues that need to be resolved.

The obtained waste from agricultural production processing should be divided into waste whose storage generates additional expenses and waste that can bring profits. Until recently, waste from agricultural production processing was not considered by livestock farmers as income-generating waste. Diversifying and expanding the assortment of protein feed sources for the zootechnical sector is an ongoing issue in the Republic of Moldova. Currently, in the republic, several small and medium-sized enterprises (JSC "Azamet" PRO) specialized in producing pumpkin seed oil with and without shells. The by-product obtained from cold-pressing pumpkin seeds for oil extraction is referred to as "oilcake" or "cake" from pumpkin seeds. It has a complex chemical composition and high nutritional value (Svezhentsov & Korobko, 2023). Available sources from the literature indicate that 100 grams of pumpkin seeds contain: 26 kcal, protein, fats, fibers, 16 mcg folate, 6 mg niacin, 2 mg pantothenic acid, 0.6 mg pyridoxine, 11 mg riboflavin, 0.5 mg thiamine, Vitamin A, Vitamin C, Vitamin E, Vitamin K, 1 mg sodium, 21 mg calcium, 340 mg potassium, 44 mg phosphorus, 262 mg magnesium, 515 mcg alpha-carotene, 3100 mcg beta-carotene, and 8 types of essential amino acids (Butch, 1983; Vasilyeva et al., 2010). Literature data suggest that, following the cold pressing of pumpkin seeds, up to 10% of nutrients are found in pumpkin seed oil, and the main part of nutrients is present in the seed cake (Coshman et al., 2023). Regrettably, scientific and specialized literature provides very limited or entirely missing information about the use of pumpkin seed cake in animal feed (especially for pigs). Based on the mentioned facts and considering the periodic occurrence of severe droughts in the Republic, often compromising cereal crops (corn, barley, wheat, soybeans, peas, etc.), the purpose of the research was to study the chemical composition and nutritional value of pumpkin seed cake. Additionally, the study aimed to assess the impact of using pumpkin seed cake in the diet of fattening young pigs on production performance, blood indicators, and economic factors.

MATERIALS AND METHODS

The investigations were conducted in the laboratories of the Scientific-Practical Institute of Biotechnologies in Zootechnics and Veterinary Medicine, as well as within the unit of reproduction, growth, and fattening of pigs IE "Secrieru Aliona" in Stoianovca village, Cantemir district, Republic of Moldova. The subject of the research was pumpkin seed cake with shells and three-way crossbred piglets (Yorcshire x Landrace x Duroc), selected using the batch-analogue method, considering their origin, body weight, health status, and growth potential during the leveling period (Table 1).

Table 1. Experimental design scheme

Lot	Livestock (n)	Feeding particularities
The first period of fattening 40-70 kg		
Control	12	NCB* - (recipe 1)
Experimental	12	NCE** - (recipe 2)
Second fattening period 71-125 kg		
Control	12	NCB* - (recipe 3)
Experimental	12	NCE** - (recipe 4)

Note: NCB* - standard combined feed;
NCE** - experimental compound feed.

The research focused on studying the chemical composition and nutritional value of pumpkin seed cake and the combined fodder, morphological and biochemical parameters of blood, production performance, and economic indicators depending on the proportion of pumpkin seed cake in the rations.

In the recipes of combined fodder from experimental groups, the pumpkin seed cake had a participation rate of 4% (Recipe 2) and 7% (Recipe 4), while the quantity of soybean meal was reduced in the same proportions.

The combined fodder recipes for each fattening period were developed using the computerized program "HYBRIMIN," following current nutritional standards (Kalashnisov et al., 2003), and prepared in the feed production section of the designated enterprise. The chemical composition of pumpkin seed cake and the combined fodder used in the experiment was assessed using classical methods (Lebedev & Usovich 1976; Petukhova et al., 1989).

Animals selected were raised under the same spatial and microclimate conditions, with ad libitum feeding and daily recording of consumption. Access to water was provided continuously.

Animals were selected using classical methods (Ovsyannikov, 1976). At the beginning and end of the experiment, blood samples were collected from three piglets in each group and analyzed using the STAT FAX-3300 biochemical analyzer.

The chemical composition of the meat (water, protein, fat, collagen) from the Longissimus dorsi muscles was evaluated using the computerized program "Scanlab NIT 98".

Biometric data processing and testing the significance of differences were conducted using the computerized program EXCEL, employing classical methods (Plokhinsky, 1978).

RESULTS AND DISCUSSIONS

Pumpkin seed cake with shells (residue obtained from cold-pressing pumpkin seeds for oil extraction) was acquired from ISC "Azamet" Pro, Ciadir-Lunga, Republic of Moldova. Based on organoleptic analysis, it was determined that the pumpkin seed cake with shells consists of granules of various sizes, exhibiting a range of colors from brown to light gray with greenish shades characteristic of pumpkin seeds (Figure 1). It has a pleasant cereal-like aroma and a subtly sweet taste without any foreign tastes.

Figure.1 Pumpkin seed cake with crust

As a result of the chemical composition analysis, it was determined that pumpkin seed cake with shells is a valuable protein supplement containing up to 40.5% crude protein, with a significant proportion of crude fiber at 28.07%. Pumpkin seed cake obtained through cold-pressing contains: nutritional units - 1.06; metabolizable energy - 11.21 MJ; crude protein - 40.5%; crude fat - 14.41%; crude fiber - 28.07%; non-nitrogenous extractive substances - 1.21%; calcium - 0.09%; phosphorus - 0.21%; with a sugar content of 2.29% and carotene content of - 4.50 mg/kg. According to current nutritional standards and the experimental design, using local ingredients, two new combined fodder recipes were formulated and tested for the fattening period (Table 2).

Table 2. Structure of the combined fodder recipes used in the experiment (%)

Ingredients	Fattening period			
	40-70 kg		71-125 kg	
	Control	Experim.	Control	Experim.
Corn	20.0	20.0	20.5	20.5
Barley	13.5	13.5	30.0	30.0
Wheat	30.0	30.0	23.5	23.5
Wheat bran	4.0	4.0	4.5	4.5
Soybean meal	17.0	13.0	12.0	5.0
Sunflower seed cake	6.0	6.0	4.0	4.0
Pumpkin seed cake	-	4.0	-	7.0
Fish meal	3.0	3.0	-	-
Premix	3.0	3.0	3.0	3.0
Zeolite	3.5	3.5	2.5	2.5
Total	100	100	100	100

According to the results of the chemical composition analysis, the nutritional value of 1 kg of combined fodder used during the 40-70 kg fattening period corresponded to the following values for the groups: crude protein - 175.20; 179.24 g/kg, metabolizable energy - 10.44; 10.75 MJ/kg, crude fat - 52.08; 52.83 g/kg, crude fiber - 55.32; 42.31 g/kg, non-nitrogenous extractive substances - 53.41; 55.79%, calcium - 0.79; 0.61%, nutritional units - 0.87; 0.90%. For the 71 kg to slaughter fattening period, the values were: crude protein - 153.96; 152.46 g/kg, metabolizable energy - 10.90; 10.73 MJ/kg, crude fat - 58.41; 79.09 g/kg, crude fiber - 70.08; 101.6 g/kg, non-nitrogenous extractive substances - 57.25; 48.50%, calcium - 0.43; 0.47%, nutritional units - 0.91; 0.89%. These values complied with nutritional standards (Kalashnisov, 2003).

The biological test was conducted at the reproduction, growth, and fattening unit IE "Secrieru Aliona" in Stoianovca village, Cantemir district, Republic of Moldova, involving 24 three-way crossbred piglets (Yorkshire x Landrace x Duroc) over a period of 112 days, with the first fattening period (40-70 kg) comprising 50 days and the second fattening period (71 kg to slaughter) lasting 62 days. The test animals were randomly allocated into two homogeneous groups, each with 12 piglets, with an initial average weight per group of 40-41 kg.

Replacing soybean meal with pumpkin seed cake at proportions of 4% and 7% did not significantly affect the feed intake, and the average daily feed consumption had values of 2,934 kg and 2,944 kg, respectively, corresponding to the groups.

The study of the live weight dynamics revealed that the animals in the control group had a slower growth rate compared to those in the experimental group and exhibited lower average daily weight gain rates.

During the testing period, the live weight obtained was 86.25 kg/head for animals in the control group and 88.42 kg/head for animals in the experimental group, representing an increase of 2,170 kg (2.52%) in the experimental group (Table 3).

Table 3. Evolution of live weight dynamics and daily weight gain

Specifics		Lot	
		Control	Experimental
Live weight, kg	at the beginning of the experiment	39.75±0.809	41.42±0.552
	end of I growth periods	72.75±0.577	74.58±0.623
	end of experience	126.00±0.932	129.00±1.205
Growth increase, kg	in the first period	33.00±0.532	31.17±0.343
	in the second period	53.25±1.182	55.25±1.256
	in the experiment	86.25±1.311	88.42±1.049
Average daily gain, g	in the first period	660±9.621	663±6.210
	in the second period	859±19.060	891±20.263
	in the experiment	770±11.709	789±9.365
Feed consumption, kg per 1 kg of gain		3.810	3.730

The best results for average daily gain in the second fattening period were observed in the experimental group with 891 g, representing a 3.73% increase compared to the control group with 859 g. It's worth noting that over the 112 days of the experiment, the average daily gain in the control group was 2.3% lower than in the experimental group. The best feed conversion ratio was achieved by piglets in the experimental group II, which was by 80 g lower than in the control group, representing a 2.1% improvement.

It was determined that the use of pumpkin seed cake in the diet of fattened piglets in various proportions did not have a negative impact on blood parameters.

Based on the results of blood parameter analysis at the end of the experiment, several positive points regarding the studied parameters in the groups of piglets under study can be mentioned. The total protein content in the blood serum of piglets in the control group increased from 62.20 g/l at the beginning of the test to 82.49 g/l at the end of the test, an increase of 20.29 g/l. In the experimental group, the total protein content increased from 68.78 g/l to 81.77 g/l, an increase of 12.99 g/l.

It is noteworthy that at the end of the experiment, the albumin content showed small fluctuations and ranged from 22.83 g/l to 18.57 g/l, with deviations within the acceptable physiological norms (20-60 g/l). In our research, the amount of uric acid in the blood, an indicator characterizing the activity of renal functions, ranged from 5.27 mmol/l in the control group to 3.25 mmol/l in the experimental group, falling within the permissible physiological norms (2.8-8.8 mmol/l). The study of blood serum indicators demonstrates that the combined fodder recipes used in the experiment do not significantly alter the blood parameters (Figure 2). This suggests normal functioning of all organs and systems in the animals from both groups.

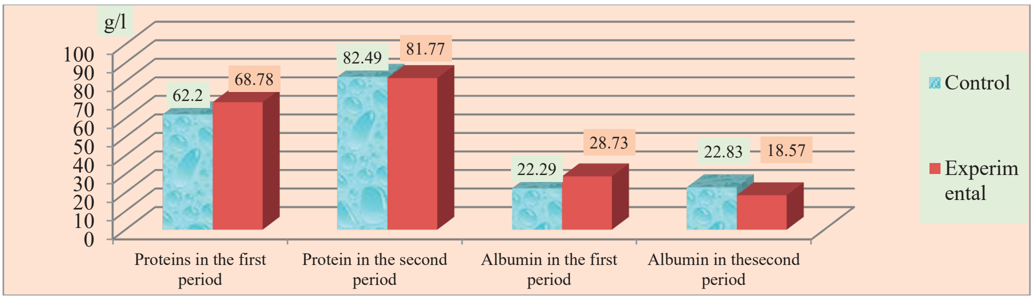

Figure 2.The amount of protein and albumin in the blood serum (g/l)

In the objective assessment of pig carcasses, slaughter yield represents a criterion that is highly important both quantitatively and qualitatively. We found that sows in both groups achieved good carcasses with a relatively high slaughter yield (hot carcass weight) of 78.10% in the control group and 80.53% in the experimental group. According

to this index, the animals in the experimental group exceeded the control group by 2.43%.
The main characteristics influencing carcass quality in pigs are the thickness of the dorsal fat layer, carcass length, and the proportion of meat, especially high-quality meat, which is closely related to the surface of the Longissimus dorsi muscle eye (Dinu et al., 1993). According to the results obtained, there is a trend of reducing the thickness of the back fat layer at the 6/7 thoracic vertebra by 4 mm and the spine by 2 mm in the carcasses of sows from the experimental group. Both groups of sows showed a uniform deposition of thin fat at the rump, with an average of 18.67 mm (Table 4).

Table 4. Formation of fat layer thickness (mm)

Carcass region	Lot	
	Control	Experimental
6/7 thoracic vertebra	33.33±3.342	29.33±2.483
Spine	25.00±3.674	23.00±2.550
Hams	20.33±0.408	21.00±1.871
Rump	18.67±0.816	18.67±1.633
Chest	19.00±0.707	21.67±2.858
Abdomen	13.67±2.677	14.66±0.408

Among the dimensional determinations, the large length of the carcass is important. The larger it is, the more extended loin and hams we will benefit from. The measurements of the large length (Table 5) demonstrate that the carcasses of animals in the experimental group were by 1.2 cm longer than those in the control group.

Table 5. Main measurements of sacrificed pig carcasses (cm)

Indices	Lot	
	Control	Experimental
Lomg length	128.8±1.814	130.0±3.742
Short length	97.3±0.965	97.2±0.736

Regardless of the administered recipes, both in the control and experimental groups, the small length of the carcasses was almost the same, with an average value of 97 cm.
It is well known that in increasing pork production, an important role is played by the development and appreciation of the Longissimus dorsi muscle. Based on the measurements and calculations performed, it was established that all sacrificed piglets produced long carcasses with a muscle eye area of 41.98 cm² in the control group and 42.69 cm² in the experimental group, as shown in Figure 3.

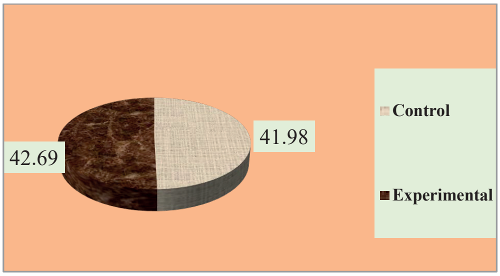

Figure 3. Eye muscle area (cm²)

The muscle eye area was larger in the experimental group by 1.69%.
Since the ham is a region that provides high-quality and substantial meat, our investigations confirmed that animals from both groups produced heavy hams, weighing 11,567 kg and 11,767 kg, respectively. The hams of piglets from the experimental group were heavier by 200g compared to those from the control group, representing a 1.73% increase (Figure 4).

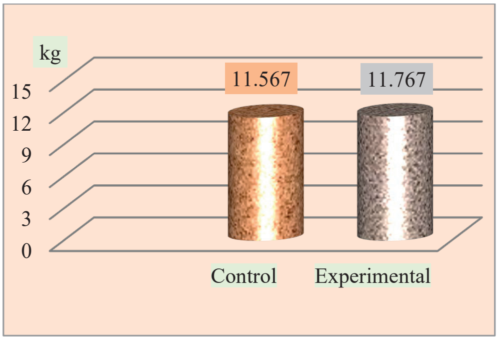

Figure 4. Ham weight (kg)

In the muscle tissue of sacrificed animals, the water, protein, and collagen content varies within relatively narrow limits. Animals that consumed a feed mix containing pumpkin seed cake showed a tendency to increase the fat content, providing the meat with tenderness, juiciness, and high energy value. The meat of piglets from the experimental group recorded an increased fat content of 4.5%, surpassing the control group by 0.70% (Figure 5).

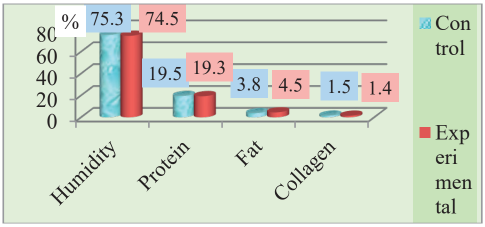

Figure 5. Chemical composition of meat (%)

The economic efficiency of using pumpkin seed cake in pig feeding was calculated based on the absolute weight gain of the animals during the experiment, the cost of 1 kg of absolute weight gain, the consumption of combined fodder, the cost of combined fodder, and the cost of pumpkin seed cake with shells.
It was determined that the use of pumpkin seed cake with shells reduces the cost of 1 kg of combined fodder during the growth-fattening period by 9.4 cents (2.7%) and 16.7 cents (6.2%) during the finishing period.
Substituting soybean meal in combined fodder recipes with pumpkin seed cake at proportions of 4% and 7% resulted in an economic benefit per animal, with a weight gain effect of 5.88 euros per head and a reduction in the cost of combined fodder of 4.74 euros per head.
The use of pumpkin seed cake in pig diets led to an economic benefit of 10.62 euros for each pig raised and fattened.

CONCLUSIONS

Based on the obtained data, we can conclude that pumpkin seed cake with shells is a valuable protein supplement. Due to its good organoleptic qualities, it can be positioned as a high-ranking source of plant-based protein and can be accepted for use in the feeding of growing and fattening pigs.
The tests showed that substituting soybean meal with pumpkin seed cake at proportions of 4% in the first period and 7% in the second fattening period provides an average daily weight gain of 789 g, slaughter yield (hot carcass weight) of 80.53%, backfat thickness at the 6/7 thoracic vertebrae of 29.33 mm, at the spine of 23.00 mm, at the rump of 18.67 mm, eye muscle area of 42.69 cm², ham weight of 11,767 kg, and fat content in the *Longissimus dorsi* muscle of 4.53%.
The research results indicate that pumpkin seed cake with shells is a viable solution for the partial replacement of soybean meal in the diets of fattening pigs, as it reduces the cost per kg of combined fodder by 2.7% in the first period and 6.2% in the second fattening period, resulting in an economic benefit of 10.62 euros for each piglet raised and fattened.

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NONCONVENTIONAL RESOURCES FOR MONOGASTRIC FEEDING

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Abstract

Non-conventional feed resources (NCFR) as potential alternative feed ingredients for animal production continue to be a topic of great interest in recent years. As a promising solution for livestock feeding to reduce feed costs and ensure productivity and environmental sustainability with maximum efficiency, some of the potentially available NCFR are useful oilseed by-products. Some of them have a high nutritional value and contain several bioactive compounds (dietary fiber, essential oils, vitamins, minerals, polyphenols, etc.) which can promote the health and well-being of animals. However, the protein content, low energy level or the presence of some anti-nutritional factors can compromise its quality and restrict its use in animal feeding. Therefore, this paper reviews the nutritional value of three NCFR cold-pressed cakes, i.e., flax, hemp, and pumpkin) and the effects of their inclusion in monogastric diets (poultry and pigs), to provide a theoretical reference for their usefulness in the nutrition field.

Key words: *animal nutrition, cakes, flax, hemp, pumpkin.*

INTRODUCTION

The development of animal breeding by scientific progress and the increasing demands of the national economy is primarily based on finding some nutritional strategies to make animal feed more efficient. In general, the lack of animal feed resources has mobilized animal nutrition researchers to find promising solutions to ensure cheap and environmentally sustainable feedstuffs (Rakita et al., 2022). For this reason, it is important, to focus on circular economy, where the by-products from food industries have involved much more attention (Berwanger et al., 2014). Moreover, increasing the availability of non-conventional feed resources (NCFR*)* for animal livestock is an essential nutritional solution. Generally, NCFR refers to all those feeds that have not been traditionally used for feeding livestock and are not commercially used in the production of livestock feed (Areaya, 2018). Thus, in our paper, the NCFR term has been used to describe different sources such as oil seeds by-products with applicability in animal consumption (Amata, 2014). In this sense, one alternative solution is based on reducing food waste and trying to extract the maximum values from their by-products (Rakita et al., 2022). Instead, NCFR contains high amounts of nutrients and bioactive compounds, such as polyphenols, carbohydrates, lipids, organic acids, proteins, antioxidants (Švarc-Gajić et al., 2020), dietary fiber, minerals, oils, essential vitamins (Malenica et al., 2022), which could provide numerous benefits on animal welfare, performance, and health status (Manju Wadhwa et al., 2015). Nowadays, more and more attention has been paid to the by-products as a result of oilseeds extraction from oleaginous plants. Oil can be extracted by applying pressure and shear forces with mechanical expeller presses in a process called "*pressing*" (Zheng et al., 2003; Shim et al., 2015; Vidal et al., 2022). Cold pressed do not alter the crude oils (Berwanger et al., 2014). Compared with solvent extraction treatments (i.e., hexane, xylene, and toluene), the cold pressing produces a solid meal-defined *cake* with a range of oil contents, higher level of fat (6-7% *vs*. < 1%), crude protein and crude fiber (Shim et al., 2015). Due to the applied extraction conditions that cause a decrease in the efficiency of oil extraction from seeds

(Petraru & Amariei, 2020; Vidal et al., 2022), cold pressing is seen as an alternative to conventional pressing. Instead, following the Codex Alimentarius, the cold pressing is led at a temperature not exceeding 50°C (Petraru & Amariei, 2020; Sumara et al., 2023) and the oxidation stability is lower than extraction with organic solvents (Kostić et al., 2013). Further, cold pressing is cheap, rapid, and easy technique to obtain cakes even if it is used in small quantities of raw materials (Cakaloglu et al., 2018). The oilseed cakes are very rich in proteins and represent an alternative nutritional solution to cover the necessary proteins for food production, animal feed or biotechnological processes (Teh & Bekhit, 2015). Instead, that remains after oil seed extraction permits effective utilization and successful realization of the circular economy concept (Petraru & Amariei, 2020), especially in the field of animal nutrition (Petraru et al., 2021). The term oilcake is synonymous with press cake, meal, or oil meal (Petraru & Amariei, 2020), which can be used successfully in livestock production. In addition, Serrapica et al. (2019) affirmed that oilseed cakes are an excellent alternative source rich in proteins providing an increase of biomass in animal diets.

This review is aimed at providing an overview of the (i) current knowledge about the chemical composition and nutritional value of three NCFR cold-pressed cakes, such as flax, hemp, and pumpkin; and (ii) the inclusion of cold-pressed oilseed cakes in monogastric animal (poultry and pigs) diets and the effects on growth, productive performance, and products quality.

MATERIALS AND METHODS

A systematic literature search and selection methodology of the relevant research articles in international databases (PubMed, Science Direct, Google Scholar, Scopus) were accessed, explored, compared, and evaluated. The literature search was based on articles with keywords such as flax, hemp, pumpkin, cakes, animal nutrition, nutritional value, bioactive compounds, and circular economy. The aim of writing this bibliographic study is mainly to focus on the nutritional value of three NCFR cold-pressed cakes (flax, hemp, and pumpkin) and to discuss their bioactive contents as well as to present the principal effects of use in animal nutrition (poultry and pigs), respectively the results after processing it in diets.

RESULTS AND DISCUSSIONS

Oilseed cakes, general aspects

In recent years, oilseed cakes have been used as ingredients feedstuff for animals as it was an excellent and economical source of benefits (Singh et al., 2022). Source of protein, energy, carbohydrates, and minerals, oilseed cakes were found to provide benefits and to have a potent role in animal growth and production of energy (Teh et al., 2013). Due to their potential, oilseed cakes (Shim et al., 2014; Turner et al., 2014; Singh et al., 2022) contribute to better use of resources for economic growth and to meet food demand worldwide for an increasing human population (Teh et al., 2013). They can be used as substrates or can be successfully incorporated into diets to develop new products rich in nutrients (amino acids, bioactive compounds, pigments, enzymes, vitamins). Founded on variety, conditions of growth, and extraction process, the oilseed cakes involve different physiochemical and functional properties which make them unique (Rani & Badwaik, 2021). Further, oilseed cakes are recognized as low-processed materials, but safe (Petraru & Amariei, 2020). Particular attention must be paid to the storage conditions because improper preservation can cause rancidity (unpleasant smell and taste). Based on literature data, there are two types of oilseed cakes, i.e., edible, and non-edible (Cozea et al., 2016; Singh et al., 2022). The edible categories can be included in animal consumption as feed ingredients compared with non-edible cakes that consist of toxic compounds or anti-nutritional factors (Gupta et al., 2018).

Nutrient composition of oilseed cakes

Based on the nutritional value view, the by-products resulting from the production of oils from vegetable oleaginous seeds named cakes, are successfully used with significant results in animal feed (Kolláthová et al., 2019). This review begins by summarizing and characterizing the chemical composition and nutritional value of flax, hemp, and pumpkin seed cakes, presenting the health benefits and growth performance through *in vivo* studies to maximize their potential in new feed structures for animal nutrition.

Flaxseed or **linseed** (*Linum usitatissimum* L.) is an annual herb plant, a member of the *Linaceae* family. The species is native to the eastern Mediterranean, western Asia and the Middle East, up to India. Regarding the varieties, the flax plant prefers deep moist soils rich in silt, sand, and clay (Bernacchia et al., 2014). The seeds of flax are brown to yellow and golden. The composition of flax can vary with, the growing environment, genetics, seed processing and method of analysis. Flaxseed contains various phenolic compounds such as lignans, flavonoids, phenolic acids, and tannins with high antioxidant potential (Kasote, 2013). Regarding the by-product resulting from cold press extraction, flaxseed cakes (**FSC**) still have good nutritional values. However, the research on FSC is relatively limited. FSC involves excellent functions and can be used like other ingredients as NCFR in animal livestock (Xu et al., 2022). According to the literature reports, FSC has higher nutritional content after cold press of protein (from 14-41%), fats (6-22%), ash (between 4.0-7.0), and energy (Table 1). Further, the nutritional composition of FSC differs due to the factors that are closely related to the technological process of oil extraction and environmental conditions (Cozea et al., 2016).

Hemp (*Cannabis sativa* L.) is an ancient Asian crop that has been cultivated and grown for about 10,000 years (Leonard et al., 2019). As an herbaceous plant, hemp can grow from about 1 to 6 meters tall, depending on factors such as cultivation, and environmental and agronomic conditions (Fike, 2017).

Cannabis sativa can be classified into drug type (marijuana) and nondrug type (hemp). Hemp is included in the *Cannabaceae* family with four subspecies: *sativa, indica, ruderalis* and *afghanica* (McPartland et al., 2018). Industrially, there are two different types of hemp plants, namely fiber hemp and oil hemp, which is a variety of *Cannabis sativa* distinct from the marijuana used for seeds production.

A major importance of hemp is rendered by the extraction of oil from its seeds using the cold pressing process (without the involvement of the oilseed conditioning step) with a screw or a hydraulic press, respectively the solvent extraction. Instead, cold pressing is the most used because it does not involve the use of organic solvents or heat (Teh et al., 2013). Also, the cold pressing process involves the step of cleaning the seeds, grinding them into a paste and finally applying high pressure to extract the oil. After oil extraction, it results in the defatted cake, this being a by-product rich in fibers and proteins. According to literature reports, hempseed cakes (**HS**C) have a nutritional value equal to rapeseed and can be effectively used as a feed ingredient in animal feed. At the same time, the demand for the use of cold-pressed oil cakes is increasingly sought after, thanks to the simple equipment, which can be used both on a farm and in rural areas (Roy & Deshmukh, 2019), offering extraction yields of 27-31.5% (Anwar et al., 2016).

Pumpkin (*Cucurbita* spp.) belongs to the genus *Cucurbita*, family C*ucurbitaceae* and is one of the most significant vegetable crops in Mexico, being widely cultivated in South Asia, Africa, Latin America, and the United States. Depending on the variety and climate, the pumpkin is available in many shapes, sizes, and colours. The most popular pumpkin varieties worldwide are *C. pepo, C. moschata, C. mixta,* and *C. maxima*. At the same time, pumpkin is one of the most important vegetable crops, being widely consumed on agricultural land and in certain urban regions. The pumpkin has gained special attention due to its composition and the protection generated on health, all these benefits being due to the nutritional value of its seeds (Kaur & Sharma, 2018). It is known that pumpkin seeds are the most important part, but unfortunately, they are mostly thrown away as waste. Currently, pumpkin seeds are subjected to industrial processing for the extraction of oil, being also marketed as a salty snack (Kaur & Sharma, 2018). Pumpkin is characterized by low caloric content, high concentrations of β-carotene (precursor of vitamin A) and antioxidant properties (El-Adawy & Taha, 2001). The effective use of these pumpkin by-products involves the extraction of bioactive compounds (PUFAs, antioxidants, vitamins such as carotenoids and tocopherol, minerals) and their addition in the food industry chain, but especially as feed ingredients in animal nutrition, improved the nutritional value of diets (Aziz et al., 2023). Furthermore, pumpkin seeds are known for their special issues like anti-fungal, anti-inflammatory, and anti-bacterial effects (Wang & Ng, 2003; Caili et al., 2006; Kaur & Sharma, 2018).

Chemical composition

Flaxseed is a low-cost, unconventional feed resource with a unique phytochemical composition and diverse utilization. Flaxseed oil, which is mostly obtained by pressing the seeds, is an important source of omega-3 PUFA (Shim et al., 2015). Following the extraction of the beneficial component (the oil), the secondary product known as cakes has a high nutritional value. Instead, the oilseed cakes contain a balanced ratio between protein and fat content (Table 1).

Hempseed are fruits of *C. sativa* composed of white seed and brown skin. It has a round shape, dark red-brown colour, with a diameter between 3.0 to 5.0 mm (Leonard et al., 2020). Table 1 reports data on the chemical composition of HSC as rich sources of fat and protein, the differences being generated by the variety or hybrid studied, pedoclimatic conditions, agronomic practices, and processing methods (Vastolo et al., 2021). Whole seed of *Cannabis sativa* L. is a valuable source rich in protein (25-30%), polyunsaturated fatty acids (PUFA, 75-80%), dietary fiber (approximately 4%) and phenolic compounds (House et al., 2010).

Table 1. The proximate nutritional composition of oilseed cakes

Items						
DM	**CP**	**EE**	**CF**	**Ash**	**GE**	**References**
	air dry basis, %				MJ/kg DM	
Flax						
90.30	31.60	22.10	8.40	5.40	n.d.	Ogunronbi et al. (2010)
89.70	32.20	11.50	8.60	4.90	10.78	Halle & Schöne (2013)
93.11	35.36	18.18	8.56	4.92	n.d.	The et al. (2014)
89.70	32.83	21.40	9.50	5.30	n.d.	Budžaki et al. (2018)
90.73-93.10	14.40-41.97	6.11-21.40	6.29-12.90	4.70-6.27	n.d.	Petraru & Amariei (2020)
90.60	34.10	10.20	10.50	6.30	20.70	Feedipedia.org
91.20	35.40	10.70	9.70	6.30	20.90	Feedtables.com
90.20	33.90	7.02	9.88	5.45	n.d.	Xu et al. (2022)
94.50	n.d.	n.d.	n.d.	6.19	n.d.	Kolláthová et al. (2019)
Hemp						
91.30	31.90	11.50	30.30	7.20	21.60	Feedipedia
89.20	24.80	8.90	25.10	5.80	1.80	Callaway (2004)
88.00	31.00	8.50	17.90	8.10	-	Hessle et al. (2008)
93.70	34.40	12.40	n.d.	6.70	9.50	Karlsson et al. (2010)
92.00	29.80	9.69	32.55	7.24	20.40	Šťastník et al. (2019)
92.47	32.06	9.02	32.21	5.38	n.d.	Kasula et al. (2021)
94.40	37.70	16.40	26.10	7.90	2.30	Rakita et al. (2023)
Pumpkin						
93.20	52.20	12.30	6.40	8.10	n.d.	Baia et al. (1965)
93.90	52.90	16.30	39.40	8.51	13.10	Klir et al. (2017)
94.48	28.53	37.18	21.58	6.71	20.88	Wafar et al. (2017)
92.10	61.00	13.70	2.90	8.00	22.70	Greiling et al. (2018)
92.50	38.30-62.30	9.00-36.20	23.10	7.50-8.10	20.70	Keller et al. (2021)
90.77	42.96	11.23	23.08	7.51	n.d.	Boldea et al. (2021)
88.00	63.20	13.00	5.70	9.00	22.60	Feedipedia.org

Where: DM = Dry matter; CP = crude protein; CL = crude lipid; EE = ether extract; CF = crude fiber; GE=gross energy; n.d. = non defined.

Unshelled hemp seeds contain 25-34% fat, and shelled seeds contain 42-47% fat. Interestingly, hempseeds have been identified with 181 proteins with two major storage proteins being the legumin-type globulin edestine (67% to 75%) and globular-type albumin (25% to 37%; Callaway, 2004; Aiello et al., 2016). These two proteins have different AA compositions and functional properties. Structurally, edestine is a hexamer with identical subunits that belongs to the globulin family (Docimo et al., 2014), being less soluble in water or buffer with neutral or low pH, but soluble in a basic buffer (Malomo & Aluko, 2015). Despite its low solubility, edestine is known for its high digestibility (Banskota et al., 2022). *Albumin* has fewer disulfide bonds compared to edestine (globulin), with a flexible structure with higher protein solubility and foaming capacity (Malomo & Aluko, 2015). In addition to these essential nutrients, hemp contains compounds such as plant sterols and Phyto cannabinoids, including the most abundant delta-9-tetrahydrocannabinol (THC), which is a powerful fat-soluble antioxidant with a role in appetite stimulation (Potter et al., 2008).

Pumpkin seed cakes (PSC) are a high-quality by-product rich in proteins, resulting from pumpkin oil production (Steiner et al., 2020). The chemical composition of PSC is presented in Table 1. According to the literature, the CP content recorded in PSC was between the range of 28-63% more highly than FSC (14-35%) and HSC (24-38%) confirming that it is a rich source. The ether extract (EE) from PSC ranged from 11-37%, comparatively with FSC where the level was found from 7-22%, respectively 17-33% in HSC. Overall, the nutritional composition results were within expected levels and consistent with literature data. Special attention has been paid to hemp cakes as a result of the cold pressing process, which is very rich in proteins, fibers, minerals, and biologically active compounds (Majchrzak et al., 2020).

Minerals

In terms of low mineral content, oilseeds are considered deficient, meaning they are nutritional sources of a small range of minerals (Kolláthová et al., 2019). Based on literature studies, Table 2 lists the content of relevant minerals in analysed oil seed cake sources.

Table 2. The minerals content of oilseed cakes

Items									References
Macroelements					Microelements				
Ca	P	K	Na	Mg	Mn	Zn	Cu	Iron	
g/kg DM					mg/kg DM				
Flax									
3.32-3.82	6.43-8.24	8.99-10.07	0.38-0.60	4.91-5.82	32.80-49.91	64.72-69.33	16.49-20.86	-	Ogunronbi et al. (2010)
3.61	9.52	12.41	0.34	4.41	13.72	48.60	17.75	78.60	Kolláthová et al. (2019)
4.30	9.00	11.80	0.80	5.50	40.00	68.00	19.00	175.00	Feedipedia.org
4.30	9.30	11.30	0.69	5.20	39.00	66.00	18.00	164.00	Feedtables.com
Hemp									
2.90	10.50	-	-	-	-	-	-	-	Feedipedia.org
0.12	-	0.43	0.015	0.17	7.80	2.40	0.10	-	Radočaj et al. (2014)
0.17	0.71	0.95	0.01	0.48	133	133.67	77.83	18.83	Kasula et al. (2021)
Pumpkin									
1.60	17.80	13.70	0.30	5.70	80	190	16.00	211	Feedipedia.org
0.73	10.40	7.90	0.20	4.60	38.50	49.50	11.50	73.50	Frida.fooddata.dk
0.34	15.70	5.79	0.006	5.69	49.30	113	15.40	106	Glew et al. (2006)
0.60	-	<0.005	-	6.70	-	155.10	6.37	134.60	Sobczak et al. (2020)

Where: Ca = calcium; P = phosphorus; P = potassium; Na = sodium; Mg = magnesium; Mn = manganese; Zn = zinc; Cu = copper; Fe = iron.

Oilseeds are an important source of many nutrients and minerals in animal nutrition (Das et al., 2017). In recent years, Kolláthová et al. (2019) reported that there has been an increasing interest in the utilization of organic by-products of industrial processing as feed ingredients for animal nutrition. Regarding the presence of macro elements from our oilseed cakes, the flax is mostly rich in calcium, potassium, and magnesium, followed by pumpkin where the phosphorus content is higher than flax. Radočaj et al. (2014) found a low percentage of HSP (2.40 mg/kg of DM). Further, as related to Gálik et al. (2011), Zn is very important in nutrition mainly to immunize animals. The Iron (Fe) presence, in FSC and PSC, was detected the higher content. In cuprum content a wide range from 0.10-77.83 was found in HSC *vs*. FSC where values were between 16.49-20.86, respectively 6.37-15.40 in PSC.

Amino acids contents

Amino acid (AAs) composition is an essential factor in evaluating the nutritional quality of a feed resource, along with its chemical composition and degree of digestibility. The profile of AAs, especially essential AAs (EAAs), determines the biological value of proteins. The absence of an EAAs automatically inhibits the synthesis of protein, and its deficiency diminishes it. At the same time, when it makes the feed structure, synthetic AAs such as L-lysine and DL-methionine are used to cover the requirement. According to the reports data presented in Table 3, FSCs have a good EAAs profile and can constitute a great protein source for animal feed (Panaite et al., 2017). The most abundant AAs found in FSC were glutamic acid (6.18-20.40 g 100 g-[1]), followed by arginine (3.03-9.60), and aspartic acid (3.05-9.40), while the limiting was tryptophan, methionine, cystine, tyrosine, valine, and lysine. Regarding the abundance of AA found in HSC, glutamic acid, arginine, and aspartic acid are among them (Table 4). HSC protein contains all 21 known amino acids (AAs), including the 9 EAAs that the animal body cannot synthesize (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine). Because lysine is only present in small amounts of hemp protein, it is considered a limiting AA. Thus, to cover the level of lysine, nutritionists recommend that, for example, hemp protein can be combined with pea or rice protein powder.

Table 3. Amino acids profile in flaxseed cakes (g 100 g^{-1})

Essential						
LYS	4.00	3.90	3.93-4.18	3.90	1.29	3.93-5.80
THR	3.90	3.80	4.19	3.60	1.20	3.40-3.87
MET	1.90	1.90	2.20	1.40	0.58	-
ILE	4.40	4.30	4.36-5.21	4.00	1.46	2.80-4.36
LEU	6.00	6.00	6.07	5.80	1.95	6.07-6.50
PHE	4.80	4.90	5.33	4.60	1.50	-
VAL	5.20	5.10	5.17-5.42	4.60	1.73	3.50-5.42
ARG	9.60	9.40	10.63	9.20	3.03	-
HIS	2.50	2.70	2.45	2.20	0.79	-
TRP	1.60	1.50	1.38-3.87	1.80	-	-
Total sulfated AA (MET + CYS)	-	-	-	-	-	2.50-3.80
Total aromatic AA (PHE + TYR)	-	-	-	-	-	6.30-7.95
Non-essential						-
ALA	4.70	4.70	4.59	4.40	1.46	
ASP	9.40	9.70	9.78	9.30	3.05	-
CYS	1.80	1.90	2.20	1.10	0.65	-
GLU	20.40	20.10	26.92	9.30	6.18	-
GLY	6.00	5.90	6.14	5.80	1.89	-
PRO	4.20	3.70	5.24	3.50	1.23	-
SER	4.80	4.70	5.88	4.50	1.49	-
TYR	2.50	2.40	2.94	2.30	0.93	-

References: Feedipedia.org; Feedtables.com; Prestoet al. (2011); Singh et al. (2011); Stodolak et al. (2013); Guimarães et al. (2017); Bekhit et al. (2018)

Where: LYS = lysine; THR = threonine; MET = methionine; ILE = isoleucine; LEU = leucine; PHE= phenylalanine; VAL= valine; ARG = arginine; HIS= histidine; TRP = tryptophan; ALA = alanine; ASP = aspartic acid; CYS = cystine; GLU = glutamic acid; GLY =glycine; PRO = proline; SER = serine; TYR = tyrosine.

Hydrolysis of hemp seed proteins by enzymes such as pepsin, pancreatin, trypsin and proteases leads to the release of bioactive peptides with antihypertensive, antioxidant, antiproliferative and anti-inflammatory properties (Kotecka-Majchrza et al., 2021). The percentage distribution of EAAs is like that of soybean, with the mention that they have a higher methionine content (Wang & Xiong, 2019). PSC are rich in AAs like glutamic and aspartic acid, arginine, leucine, and lysine. As can be observed, the rest of AAs are presented in low quantities (Table 5). Based on protein content, reports indicate that pumpkin seed is like soybean cake protein due to the rich content of EAA (Vinayashree & Vasu, 2021).

Table 4. Amino acids profile in hempseed cakes (g 100 g^{-1})

Essential						
LYS	3.30	1.13	2.91	3.86	1.39	5.50
THR	3.20	1.18	2.6	3.94	1.42	-
MET	2.10	0.51	2.01	2.57	0.93	1.80
ILE	3.80	0.91	3.14	4.23	1.52	-
LEU	6.10	1.93	5.11	6.86	2.47	-
PHE	4.30	1.24	3.60	4.73	1.70	-
VAL	4.60	1.13	3.84	5.58	2.01	-
ARG	10.70	4.00	3.01	11.42	4.11	-
HIS	2.50	0.73	2.56	2.72	0.98	2.50
TRP	1.50	0.27	-	1.14	0.41	-
Non-essential						
ALA	4.00	1.19	3.01	4.70	-	-
ASP	9.50	1.37	7.55	10.69	-	-
CYS	1.70	0.34	1.36	2.04	0.74	2.00
GLU	16.00	1.45	13.07	17.61	-	-
GLY	4.20	1.18	2.60	4.85	-	-
PRO	3.70	4.94	3.01	4.64	--	-
SER	4.70	3.55	3.21	5.05	-	-
TYR	3.20	0.89	2.42	3.36	-	-

References: Feedipedia.org; Eriksson (2007); Karlsoon et al. (2012); Wang & Xiong (2019); Semwogerere et al. (2020); Kasula et al. (2021)

Where: LYS = lysine; THR = threonine; MET = methionine; ILE = isoleucine; LEU = leucine; PHE= phenylalanine; VAL= valine; ARG = arginine; HIS= histidine; TRP = tryptophan; ALA = alanine; ASP = aspartic acid; CYS = cystine; GLU = glutamic acid; GLY =glycine; PRO = proline; SER = serine; TYR = tyrosine.

Table 5. Amino acids profile in pumpkin seed cakes (g 100 g^{-1})

Essential				
LYS	0.50	3.80	4.66	1.36
THR	0.30	3.10	1.39	1.04
MET	0.10	2.10	-	0.73
ILE	0.30	3.90	4.05	1.29
LEU	0.60	6.90	6.60	2.49
PHE	0.40	5.50	-	1.81
VAL	0.30	4.70	4.69	1.71
ARG	1.70	16.40	14.00	5.28
HIS	0.40	2.40	1.48	0.81
TRP	0.20	2.70	-	0.61
Total sulfated AA (MET + CYS)	-	-	1.30	-
Total aromatic AA (PHE + TYR)	-	-	10.09	-
Non-essential				
ALA	0.40	3.70	3.63	1.55
ASP	1.00	9.00	11.94	3.23
CYS	0.20	1.10	-	0.39
GLU	1.80	17.90	20.40	6.32
GLY	0.60	4.20	7.30	1.88
PRO	0.40	3.40	3.65	1.39
SER	0.50	5.20	4.90	1.79
TYR	0.30	3.90	-	1.19

References: Feedipedia.org; Glew et al. (2006); Sá et al. (2021); Frida.fooddata.dk

Where: LYS = lysine; THR = threonine; MET = methionine; ILE = isoleucine; LEU = leucine; PHE= phenylalanine; VAL= valine; ARG = arginine; HIS= histidine; TRP = tryptophan; ALA = alanine; ASP = aspartic acid; CYS = cystine; GLU = glutamic acid; GLY =glycine; PRO = proline; SER = serine; TYR = tyrosine.

Further, PSC are rich in nutrients such proteins, starch, soluble sugars, crude fibers, and bioactive compounds carotenoids) which can provide sufficient energy and antioxidants for animal feed (Chen et al., 2019; Lyu et al., 2021). Li et al. (2022) stated that pumpkin has a series of benefits generated by their seeds, through the lens of nutritional characteristics, thus representing a valuable resource for animals.

Fatty acids contents

The fat acids (FA) content of pressed oilseed cakes determines their quality, ecological and economic efficacy, functional, technological, and special properties, respectively the principal value for consumers (Nikberg et al., 2011). Based on the analytical review of scientific and technical information, oilseed cakes can be used as feed ingredients with high nutritional value in animal nutrition (Stodolak et al., 2013). As shown in Table 6, FSC had between 7.73-16.88% saturated fatty acids (SFA), 5.67-41.72% monounsaturated fatty acids (MUFA), and a maximum of 71.85% polyunsaturated fatty acids (PUFA).

Table 6. Fatty acids composition of oilseed cakes

FA %							References
C14:0	**C16:0**	**C16:1**	**C18:0**	**C18:1n-9cis**	**C18:2n-6**	**C18:3n-3**	
Flax							
0.00	6.10	0.00	3.60	18.40	15.80	55.70	Feedipedia.org
0.03	5.60	0.08	4.40	20.20	14.70	53.80	Feedtables.com
n.d.	4.21-8.71	n.d.	3.52-8.17	22.10-41.72	19.13-44.82	33.22-54.79	Singh et al. (2011)
n.d.	9.54	n.d.	2.76	23.33	19.78	43.07	Aziza et al. (2013)
0.06	6.44	0.02	2.57	16.82	14.50	57.35	Stodolak et al. (2013)
0.07	5.99	0.07	2.25	15.71	12.66	42.81	Mannucci et al. (2019)
Hemp							
n.d.	6.70	n.d.	2.20	11.40	55.30	21.60	Feedipedia.org
n.d.	9.30	n.d.	3.80	13.10	52.50	19.10	Mierlita (2019)
0.07	4.46	0.15	1.76	8.27	59.52	15.85	Juodka et al. (2018)
0.17	8.77	0.14	2.51	13.83	56.98	14.62	Arango et al. (2021); Bailoni et al. (2021)
n.d.	8.60	n.d.	2.80	13.10	53.10	12.30	Tufarelli et al. (2023)
n.d.	6.00-9.30	n.d.	0.60-1.60	51.60-59.60	52.50-56.00	14.40-24.70	Halle & Schőne (2013); Mierliță (2018, 2019); Rakita et al. (2023)
Pumpkin							
n.d.	13.29	n.d.	6.74	27.06	51.04	n.d.	Radočaj et al. (2012)
0.23	14.83	0.015	6.68	25.81	50.88	0.18	Bardaa et al. (2016)
0.15	12.90	0.17	4.48	34.40	44.40	1.80	Klir et al. (2017)
0.12	13.20	0.15	4.95	29.60	49.00	0.63	Keller et al. (2021)
0.12	12.30	0.16	5.16	28.75	51.18	0.49	Boldea et al. (2021)

Where: C14:0 = myristic; C16:0 = palmitic; C16:1 = palmitoleic; C18:0 = stearic; C18:1n-9cis = oleic; C18:2n-6 = linoleic; C18:3n-3 = linolenic; nd = not detected.

The predominant SFA was palmitic acid (C16:0), while the predominant unsaturated FA was linolenic (C18:3n-3), followed by oleic (C18:1n-9cis) and linoleic acid (C18:2n-6). According to the literature, the SFA level found in HSC was between 6.00-11.45%, *vs.* MUFA where the percentage was noted from 8.00 to 59.60%. Instead, the PUFA of HSC were between 65.40-80.70% with linoleic as the predominant acid. Regarding the SFA from PSC, the level was higher (17.58-21.74%), while MUFA was between 25.82-35.70% and the PUFA was representative (46.20-51.67%). As had been was linoleic, respectively oleic acid from the MUFA profile. The results from Table 6, show that the oilseed cakes are characterized by a low quantity of SFA, of which palmitic acid is the most abundant. HSC, FSC and PSC are a major source of MUFA, where oleic acid is considered necessary. Instead, as a source of PUFA, the FSC are major, followed by PSC and HSC, where linoleic (LA) and α-linolenic acid (ALA) are considered essential. Further, these FAs are essential for health and are vital for inclusion in the animal diet due to the body incapacity of synthesising them (Rakita et al., 2023).

Benefits of oilseed cakes in monogastric feed

Alternative fodders intended for monogastric animals' production are divided into those rich in crude proteins, and respectively rich in energy (Steiner et al., 2020). In the field of animal nutrition, oilseed cakes would give potential benefits when it is utilized as a substrate converting the feed into a new beneficial product (Sarkar et al., 2021). The present report realised to compress the principal properties of oilseed cakes and the capacity of their valorisation in monogastric feed due to bioactive compounds (Figure 1).

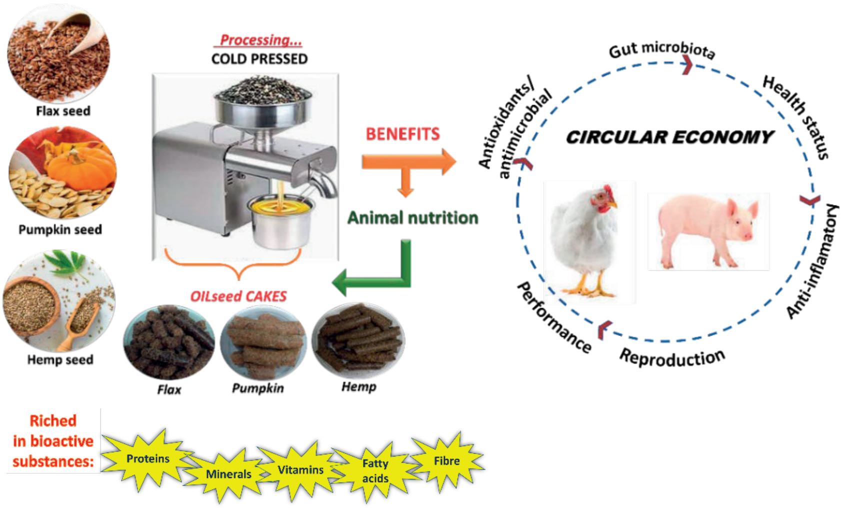

Figure 1. The schematic diagram shows various resources of NCFR rich in bioactive compounds used as monogastric animal feed (Original)

Studies on the use of FSC, HSC and PSC in the feed are relatively limited. Table 7 summarizes available reports and the main effects when cold-pressed oilseed cakes are included in poultry and pig diets.

Table 7. Effect of cold-pressed cakes in monogastric diets

Cold pressed cake	Species	Trial duration	Level of inclusion (g/100 g feed)	Main effects	References
			Poultry		
FSC	Laying hens	6 months	10%	Enrichment of yolk fat with PUFA. The egg mass production was lower.	Halle & Schöne (2013)
FSC	Broiler	42 days	5%; 10%; 15%; 20%	Improvement of carcass weight. Non-significant differences in the weights of giblets (liver, gizzard, spleen, and heart).	Leghari et al. (2017)
FSC	Layer birds	4 weeks	2% FSC +pyridoxine	Improved the omega-3 from eggs. A positive effect on egg cholesterol and health status.	Khan et al. (2019)
FSC	Duckling	3 weeks	5%; 10%; 15%	Decreased the relative weight of the left breast.	Zhai et al. (2019)
FSC	Laying hens	27 weeks	10%	Positive effect on nutrients of omega-6/omega-3 from eggs.	Perić et al. (2019)
FSC	Laying hens		Up to 26%	No effects on egg production, quality, and fertility of eggs.	Tamasgen et al. (2020)

FSC	Laying hens	6 weeks	5%	Beneficial effects on egg nutritional quality. Decrease in significant SFA content, as well as the ΣSFA/ΣUFA ratio and the PUFA ω6:ω3 ratio.	Panaite et al. (2020)
FSC	Laying hens	27 weeks	10%	Higher amounts of ALA, DHA, and PUFA in egg yolk.	Perić & Drinić (2021)
Pigs					
FSC	Growing pigs (31± kg)	12 weeks	5%; 10%; 15%	Feeding up to 15% FSC for 8 weeks had no impact on live animal performance. Feeding any level of flax for 12 weeks reduced average daily gain but feeding higher levels of flax improved feed efficiency.	Juárez et al. (2010)
FSC	Growing pigs (60 kg)	46 days	2.5%	Significantly impacts carcass FA profile.	Đorđević et al. (2016)
FSC	Growing pigs (25 kg)	28 days	12%	Reduced fat digestibility and serum cholesterol.	Ndou et al. (2017)
FSC	Pigs (60-100 kg)	40 days	5%	Muscle depth and the average meat yield was improved. PUFA and meat quality were improved.	Vlaicu et al. (2019)
			Poultry		
HSC	Laying hens	12 weeks	10%; 20%	Higher egg weights. Fatty acid profile of eggs was improved.	Gakhar et al. (2012)
HSC	Laying hens	6 months	5%; 10%; 15%	Registered lower performance. The level of SFA and MUFA decreased, while the content of LA and ALA increased with increasing dietary levels. HSC with linoleic acid oil resulted in the highest yolk fat content of this FA.	Halle & Schöne (2013)
HSC	Hens	30 days	25%	Rapport n-3 PUFA and the n-3/n-6 ratio were improved with decreasing SFA in the yolk of eggs stored at room temperature for 30 days.	Raza et al. (2016)
HSC	Laying hens	10 weeks	20.3%	No effect on growth performance. The atherogenicity index and cholesterol level were not affected. ALA, EPA, and DHA in eggs increased.	Mierlita (2019)
HSC	Growing broilers	48 days	5%; 10%	Positively influenced the lipid profile of meat. Improved the oxidative status and gut health of broilers.	Tufarelli et al. (2023)
HSC	Quails	42 days	10%	Increased n-3 PUFA content from meat. No effect was observed on performance.	Juodka et al. (2023)
HSC	Laying hens	4 weeks	5%; 10%; 20%	Egg production, feed consumption, feed efficiency, and body weight were not affected. A higher level of HSC determined a decrease in palmitic acid concentration.	Silversides & LefranÇois (2023)
			Pigs		
HSC	Growing pigs	14 days	28%	Dietary treatment affected the ileal apparent of essential AA and non-essential AA.	Presto et al. (2011)
HSC	Sows	21 days	5%	Increased n-3 FAs profile; Decreased n-6/n-3 in colostrum and milk; Increased milk yield.	Habeanu et al. (2018)

			Poultry		
PSC	Broiler chicken	49 days	10% as partial replacement of soya bean	The productive performance and meat sensorial quality were not affected.	Martínez et al. (2010)
PSC	Laying hens	49 days	3.3%; 6.6%; 10%	Increase the body weight and egg quality. Abdominal fat and serum levels of harmful lipids were reduced, while the serum levels of beneficial lipids increased.	Aguilar et al. (2011)
PSC	Chicks	56 days	5%; 10%; 15%; 20%	As the levels of PSC the growth, final body weight, total body weight gain and average daily weight gain significantly was improved.	Wafar et al. (2017)
PSC	Fattening chickens	135 days	7%; 14%	Positive effect on the production indicators and the mortality rate of chickens.	Klarić et al. (2018)
PSC	Broiler chickens	6 weeks	7%	Improve production and slaughter indicators (live, carcass, dressing percent, drumstick, breast, wings, back weight, neck)	Steiner et al. (2020)
Pigs					
PSC	Fattening pigs (60-100 kg)	40 days	5%	Improve the meat quality.	Vlaicu et al. (2019)
PSC	Fattening pigs (12-120 kg)	189 days	15% (100 g per animal)	The quality of the meat was similar but the animals were generally in good health; which also leads to the reduction of agricultural waste.	Stahn et al. (2023)

Where: FSC = flax seed cakes; HSC = hemp seed cakes; PSC = pumpkin seed cakes

Flaxseed cakes

The application of FSC in poultry diets is shown in Table 7. Particularly attention was focused on egg production. According to the literature, the addition of FSC to laying hens diets could improve n-3 PUFA content in eggs (Mattioli et al., 2017). Further, FSC is an important high-quality protein source (Xu et al., 2022), the reason for which it attracted special attention due to the presence of ALA as one of the omega-3 PUFA. Additionally, this flax by-product involves benefits in poultry due to their active compounds such as AAs, lipids, soluble fibres, and phenols. Depending on the oil extraction method, the FSC displays modifications on the chemical composition and value of the press cake. Therefore, published data on the inclusion of FSC on pigs comparatively with sow diets are more consistent. As previously reported, FSC is the richest oilseed source of n-3 PUFA and their utilisation in pig diets is necessary to increase n-3 PUFA levels from meat (Nguyen et al., 2003). Later, an inclusion of 15% FSC in finishing pig diets determined significant results on carcass FA profile (Eastwood et al., 2009). In conclusion, eggs and pork can be positively enriched with n-3 FA in a diet with a relatively low content of FSC, due to the presence of anti-nutritional factors that can affect the quality of the finished product.

Hemp seed cake

The use of HSC in diets of monogastric animals is attracting increasing interest. A peculiarity of hemp cakes is the content of narcotic substances, which is why their inclusion in nutrition diets must be done very carefully. In addition, the less dense structure of them confirms the degree of hygroscopicity; however, to date, there are limited studies in the literature (Lanzoni et al., 2024). As shown in Table 7, the use of HSC has been widely explored in poultry feed, especially in laying hen's sector. Aspects such as nutritional and functional properties of eggs by evaluating quantitative and qualitative characteristics together to zootechnical animal performance have been investigated. The inclusion of HSC in poultry feed implies several positive effects,

most likely, these results are associated with the presence of bioactive compounds (phytosterols and antioxidants) that are responsible for preventing lipid peroxidation of eggs during storage.
Regarding HSC inclusion in the swine sector, it is still in an early stage. The use of these by-products was carried out in growing pigs or lactating sows, less in piglets' diets due to the presence of stress factors generated by the weaning crisis period (Dumitru et al., 2020). Further studies are necessary to identify the best optimal level of HSC to ensure performance and animal health status.

Pumpkin seed cake
Interesting results were reported at PSC inclusion in poultry. As an alternative ingredient feed with a high potential content of protein, PSC is a good source with successful results in poultry diets (Wafar et al., 2017). Steiner et al. (2020) affirmed that PSC contains up to 500 g/kg of CP and 70 g/kg of raw fiber. As the level of PSC increased, the broiler performances significantly improved. These results can be attributed to the balance in nutrient composition and maybe to the metabolism process (Wafar et al., 2017). Instead, a dietary inclusion of 10% PSC in broiler diets determined a better feed conversion ratio (FCR) which was associated with absorption, digestion, and nutrient utilisation (Wafar et al., 2017). Research on PSC in pig nutrition has been very little studied, however, it has potential as animal feed not only for its nutritional value but also for its antioxidants, pigments, and polysaccharides content that could enhance the quality of meat, milk, and egg, as well animal health. Stahn et al. (2023) mention that PSC has a protein content of 52.1%, 6.3% fiber and 1.9% ADF. In the experiment carried out for 189 days on 3 groups of piglets (control group, BSG 1 or BSG 2) aged 42 days, they used brewer's spent grains (BSG)-raw matrix was technologically and functionally improved by adding natural active ingredient carriers (crushed wheat, rapeseed, and pumpkin seed press cake) and using planetary roller extrusion and used as feed additive for pigs. Two BSG-extrudates containing 30% BSG silage, 55% crushed wheat, and 15% rapeseed press cake (BSG 1), or 15% pumpkin seed press cake (BSG 2) were used. The quality of the meat was similar but the animals were of good health and marketable quality. BSG and agricultural residue-based feeds can be used in pig diet, which leads to the reduction of agricultural waste and reduced feed consumption that would also be suitable for human nutrition. Schwediauer et al. (2021) studied the feed intake behaviour of piglets during a 6-week suckling period. It is known that early contact with plant-based feed (creep feed) should stimulate the adaption of the gastrointestinal system and promote gut development, with the desired effect of less physiological stress at weaning, lower incidence of diarrhoea and higher growth rates due to better feed efficiency. It is also important to consider the food preferences of the piglets. In this sense, they mention the use of PSC, % in creep feed (pelleted) for piglets was 4.7%.

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CONCLUSIONS

This paper reviews the nutritional value of three NCFR cold-pressed cakes based on flax, hemp, and pumpkin and the effects of their inclusion in diets for monogastric animals (pigs and poultry), to provide a theoretical reference for their usefulness in the nutrition field.

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EFFECT OF DIHYDROQUERCETIN ON PERFORMANCE, BACK FAT THICKNESS AND BLOOD BIOCHEMICAL INDICES IN FATTENING PIGS

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Abstract

The study aimed to investigate the effect of gradient levels of 100 mg and 200 mg dihydroquercetin DHQ/kg feed added on performance, back fat thickness, and blood biochemical indices in fattening pigs. An experiment with 30 pigs of the Danube White breed with an initial live weight of 66.3 - 66.5 kg and a final live weight of 100.9 - 102.8 kg, randomly assigned to three treatments – control(C), DHQ1 and DHQ2, was carried out. Pigs were housed individually for 43 days. At the end of the experiment, the thickness of the back fat was measured, and blood samples were taken. Biochemistry indices and fat metabolism indices were studied. Administration of dihydroquercetin did not affect parameters of pig performance in fattening period. The addition of two consecutive levels of DHQ increased MLT, measured in vivo, linearly (P=0.025). The blood glucose content was linearly reduced (P<0.05). A statistically significant effect on high-density lipoproteins (HDL) in animals treated with 200 mg of DHQ (P=0.012), having a high linear dependence (P=0.007) was found, and a trend to reduce the content of triglycerides in the blood of fattening pigs.

Key words: *ADG, back fat thickness, biochemical indices, dihydroquercetin, fat metabolism, FCR, pigs.*

INTRODUCTION

Dihydroquarcetin (DHQ, also known as Taxifolin) is a powerful natural antioxidant and capillary protector related to bioflavonoids with P-vitamin activity. As a substance with a high degree of biological activity, DHQ has a whole range of positive (pleiotropic) effects on metabolic reactions and the dynamics of various pathological processes, which were identified in a number of studies by Russian and foreign scientists, in particular, in terms of antioxidant, radioprotective, membrane-protective, capillary-protective, angioprotective, lipid-lowering, anti-inflammatory, anti-allergic, cardioprotective, hepatoprotective, detoxifying, neuroprotective, gastroprotective, immunomodulatory, retinoprotective, endocrinological properties (Fomichev et al., 2017; Sunil & Xu, 2019; Liu et al., 2023). DHQ is known for preventing stress syndrome and chronic fatigue, restoring and improving the state of the body under high physical and psycho-emotional stress (Plotnikov et al., 2005).

The introduction of DHQ in the feeding of farm animals and poultry has a positive effect on immunodeficiency, broncho-pulmonary diseases and disorders in the functional state of the liver and other organs, which are usually a consequence of the impact of adverse environmental factors and modern breeding technologies, inadequate to the physiology of farm animals. Positive effects as improving productivity, survival, food safety, reducing the incidence of animal diseases, normalizing metabolic processes in the body and the functional state of the liver have been noticed when DHQ take place in animals' diets (Nikanova & Fomichev, 2012; Bogolyubova et al., 2019; Zou et al., 2016b). Dihydroquercetin is interesting for pig breeding as adaptogen positively affecting the antioxidant status of animals (Semenova et al., 2020). It was established that the use of dihydroquercetin in pig nutrition blocks lipid peroxidation processes throughout the growing and fattening period (Fomichev et al., 2017). In another experiment, it was found that quercetin attenuated oxidative stress and reduced intestinal inflammation, while reducing the number of reactive oxygen species and malondialdehyde in the intestine, endotoxins in the blood serum, and increasing the height of jejunal villi (Zou et al., 2016a). Recently, a series of new 3-monoacylated dihydroquercetin derivatives with enhanced antioxidant properties have been synthesized (Knyazev et al., 2018). Dihydroquercetin can affect lipid metabolism by regulating enzyme activity, reduce hepatic fat synthesis, inhibit intracellular cholesterol synthesis, and inhibit cholesterol esterification, triacylglycerol and phospholipid synthesis (Ren et al., 2021; Wang et al., 2022).

There is no consensus on the effective doses of DHQ for effects on the organism of farm animals. Both insufficient data and differences exist in the literature. We worked with doses of 3.5 mg/kg and 7.5 mg/kg live weight in order to improve the quality of the meat by enriching it with 95% purified biologically active additive DHQ (Ivanova et al., 2021a). Kuzmina et al. (2021) applied DHQ, produced by the Russian company Ametis, according to their recommendation instructions, to broiler chickens, in amounts of 0.50 g, 0.75 g and 1.00 g per 100 kg of feed, with the best results in terms of fattening and slaughtering qualities at the dosage of 1 g of DHQ per 100 g of feed. At the same time, Pirgozliev et al. (2021), applying DHQ to broiler feed diets at doses of 0.5 g, 1.5 g and 4.5 g per 1 kg of feed, concluded that it could be beneficial at levels, greater than 1.5 g/kg feed, due to the improved antioxidant status of the birds.

The aim of the present study was to test the effect of two gradient levels of dihydroquercetin on weight development, back fat thickness and biochemical parameters in fattening pigs.

MATERIALS AND METHODS

One trial with total 30 pigs of the Danube White breed was carried out in the Experimental Unit of the Agricultural Institute - Shumen, randomly assigned into three group, as follows:

1. Control group (C) - 10 pigs, without added biologically active component to the feed;
2. Experimental group 1 (DHQ1) - 10 pigs, with added 100 mg dihydroquercetin/kg feed;
3. Experimental group 2 (DHQ2) - 10 pigs, with added 200 mg dihydroquercetin/kg feed.

Increasing doses of DHQ formulated as gradient levels were used, with two values, to determine whether there was a linear relationship on weight and biochemical parameters.

Table 1. Component composition and content of energy and nutrients in 1 kg of compound feed

Components	Persentage (%)
Maize	13.00
Barley	10.00
Wheat	50.00
Wheat brains	7.00
Bioconcentrate BC14*	25.00
Total:	100%
1 kg of compound feed contents:	
Digestible energy, MJ	13.72
Crude protein, %	15.70
Lysine, %	0.72
Calcium, %	0.86
Phosphorous, %	0.60

*Bioconcentrate BC14 contents: 312.10 g/kg crude protein, 10.70 g/kg crude fats, 153.00 g/kg crude ash, 38.10 g/kg crude fibers, 5.88 g/100 g lysine, 2.79 g/100 g methionine, 7.80 g/100 g calcium, 2.69 g/100 g phosphorous, 2680 mg/kg Cu sulphate, 670 mg/kg dl-α-tocopherol, 93800 UI/kg vitamin A, 16080 UI/kg vitamin D3, 1975,845 kcal/kg total energy.

The pigs for the experiment were selected and equalized by origin, age, live weight, sex, immediately after weaning. After reaching an average weight of 66 kg, beginning of finishing phase, all pigs were weighed and moved to a room with individual partly slatted pens. Pigs have a total area of 2.50 m^2 with a solid concrete part (150 x 100 cm) and slatted part (100x100 cm). All pens were equipped by an individual feeder (100 x 50 cm) and an individual nipple drinker. During the fattening period, the animals were raised according to the requirements of Council Directive 2008/120/EC laying down minimum standards for the protection of pigs. They were fed a special diet, which was analysed in the Forage Laboratory of the Agricultural Institute – Shumen. The feed of the animals was weighed individually, twice a day. Pigs from the two experimental groups received DHQ, according to the amount of feed consumed for the day, in two concentrations described above. A trial of the studying effect of dihydroquercetin started from 66 kg live weight until the fattening pigs reached about 100 kg and lasted for 43 days.

The following indicators were controlled: initial and final live weight, daily diet, feed consumption - daily, individual, total and average daily gain for the period, feed utilization, health status.

A chemical analysis was performed on diet based on corn, barley, wheat bran and Bioconcentrate. The chemical composition of the feed samples was determined according to the methods adopted in Agricultural Institute - Shumen.

Each pig was fed individually, with a daily check for the presence of residues. During the experiment, none were found. The supplement was also stretched individually for each pig according to the daily feed intake. Each daily dose of the supplement was mixed with a small amount of feed immediately after weighing and given at the same time as the animals' morning feed.

The weight development of the animals was measured in the beginning and at the end of the fattening period. The total gain, the average daily gain (ADG) and the feed consumption per kilogram of gain were calculated for each pig separately.

Back fat thickness and lean meat percentage *in vivo* were determined using a "Piglog 105" apparatus (Carometec Food Technology A/S, Kolding, Denmark). The following regression model was used:

LM=63.8662-0.4465x1-0.5096x2+0.1281x3

where:

LM - percentage of lean meat in the carcass;

X_1 - back fat thickness measured between the 3-4 lumbar ribs at 7 cm laterally (mm);

X_2 - back fat thickness measured between the 3-4 lumbar ribs at 7 cm laterally (mm);

X_3 - thickness of *m. Longissimus thoracis* (MLT) between the 3-4 lumbar ribs at 7 cm laterally (mm).

In vivo lean meat content was measured on the day of termination of the experiment when animals reached 100 kg live weight.

The health status of the pigs was monitored. Blood sampling was performed at the end of the experimental period, with vacuum containers from the *sinus ophtalmicus* in the medial corner of the eye. Immediately after blood collection, the containers were transferred to the laboratory and centrifuged to separate the serum at 3000 rpm for 15 min at 4°C. After separation of the serum, it was frozen at -20˚C. Analyses of biochemical parameters were performed in a laboratory with special porcine kits with an Olympus AU640 apparatus (Beckman/Olympus counter) according to methods approved by the International Federation for Clinical Chemistry (IFCC). Biochemical indicators of liver enzymes Alanine aminotransferase (ALAT) and Aspartate aminotransferase (ASAT) were analysed by UV kinetic method; the total protein in the blood, by photometric colorimetric method. The indicators responsible for carbohydrate and protein metabolism were also investigated: glucose content by the hexokinase glucose-6-phosphate dehydrogenase method, creatinine - by the Jaffe kinetic method and urea by the enzymatic method (Urease/GLDH).

The lipid profile was studied - contents of total cholesterol, LDL, HDL cholesterol and triglycerides in the blood serum. The content of total cholesterol and triglycerides was determined by an enzymatic colorimetric method, and LDL and HDL - by a direct method.

Statistical analysis of the results

The experiment was based on a 2 x 2 factorial design (sex of pigs x dose of dihydroquercetin). Data were analyzed using the statistical software package Genstat (21st edition) (IACR Rothamstead, Hertfordshire, UK). Comparisons between study variables were performed by one-way ANOVA analysis (with spatial blocks) followed by Duncan's multiple range test. All data were checked for homogeneity of variances and normality before conducting ANOVA. An orthogonal polynomial test was used to check if there is a potential linear relationship between the increase in DHQ levels and the increase in the values of the investigated parameters. In all cases, differences between groups were reported as significant at $P<0.05$

RESULTS AND DISCUSSIONS

During the experimental period, no difference in feed consumption was found both between groups and within groups between individuals (Table 2). In total, for the research period of 43 days from the end of October to the beginning of December 2021, a feed intake of 3.186 kg/pig/day was recorded. Similar feed consumption was recorded in our previous study (Ivanova et al., 2021a) - 3.095 kg/pig/day in the group consuming 7.5 mg DHQ per kg live weight per day. No statistically significant differences were found in the final live weight of the fattening pigs between the groups with different levels of DHQ tested and the control group, indicating that the intake of DHQ had no effect on the weight development of the animals. No differences were found between male and female animals and in ADG as well ($P>0.05$). In contrast to our study, growth-stimulating effect of DHQ was found in Russia when the preparation "Ekostimul-1" (containing about 80% DHQ) was administered to 4 groups of weaned pigs of the Large White breed after weaning at the age of 60 days for 30 days (Fomichev et al., 2016). In two of the experimental groups, there was an increase in ADG with a mean value of 540 g, which was 97 g or 21.8% higher than in the control group (P<0.001). Moreover, the combined use of DHQ with probiotics increased the average daily gain of suckling piglets by 21.5% (P<0.001), while reducing feed consumption per 1 kg of gain by 17.7%, reducing the number of disorders in the digestive system for the entire experimental period by an average of 45.6%, and increases the economic effect by 16.5% (Fomochev et al., 2017). The inclusion of another feed supplement with DHQ (Ekostimul-2, containing an extract of Daurian larch with 80% DHQ and 20% other antioxidants) in the diet of pigs in the post-weaning period in a dosage of 50 mg/head/day significantly weakened the effect of stress factors of the environment and increased the adaptive capacity of animals (Nikanova & Fomichev, 2012). As a result, ADG in the post-weaning period was 20.6% higher than in control individuals.

Regarding feed utilization, no statistically significant differences were found in this study. The application of DHQ resulted in a reduction of feed required to form 1 kg body mass by 4.80% in DHQ1 and by 4.22% in DHQ2 (Table 2). The values of the indicator itself were relatively high, due to the temperature in the room in the autumn-winter period. Different levels of DHQ generally had an effect on back fat thickness in this study. Small differences were observed in the thickness of the fat at point X_2, where it was the thinnest in DHQ2 - by 5.67% compared to the C and by 1.42% compared to the DHQ1. In another study carried out by us (Ivanova et al., 2021a), with the addition of DHQ to the feed of fattening pigs in an amount of 7.5 g/kg live weight, a reduction of back fat by 15.49% was found at point X_1 (P<0.05). Similarly, in a study of Yordanova et al. (2022) in fattening pigs, a 12.02% reduction in back fat at point X2 was found, when adding 7.5 g per head per day apple pectin (containing polyphenols). An effect of supplementation with active components polyphenols was also reported in our study with entire male pigs (Ivanova et al., 2021b). A 33.5% (P<0.0001) and 18.32% reduction in the point X_2 (n.s.) of the back fat were reported. This effect of dihydroquercetin is likely due to its composition. Numerous human nutrition studies have found that polyphenols have the property to affect adipose tissue, decreasing its content (Hu et al., 2020; Singh et al., 2020; Aloo et al., 2023).

Table 2. Performance and back fat thickness measurements *in vivo* (PigLog 105) at two levels of DHQ in fattening pigs

Variables	Parameters								
	Initial live weight (kg)	Final live weight (kg)	Total gain (kg)	Av. daily gain (kg)	FCR[a] (kg)	X_1[b], (mm)	X_2[b] (mm)	X_3[c] (MLT) (mm)	LM[d] (%)
Sex									
Female (F)	64.9	102.7	36.40	0.847	3.736	17.3	12.60	47.3	55.79
Male (M)	68.0	101.3	34.75	0.808	3.900	19.4	14.93	44.0	53.01
SEM	2.32	2.23	1.065	0.025	0.128	1.44	0.856	1.70	0.964
DHQ (dihydroquercetin)									
Level 0[e]	66.5	100.9	34.43	0.801	3.936	18.5	14.10	41.6	53.92
Level 1[e]	66.3	102.8	33.50	0.848	3.747	18.2	13.90	47.3	54.72
Level 2[e]	66.5	102.3	35.85	0.834	3.770	18.3	13.30	48.2	54.36
SEM	2.85	2.73	1.304	0.030	0.157	1.77	1.048	2.08	1.180
Sex.DHQ									
Fx0	68.2	102.2	34.00	0.791	4.019	17.4	12.80	43.4	55.10
Fx1	65.4	104.8	39.40	0.916	3.409	16.4	12.40	47.8	56.36
Fx2	61.0	96.8	35.80	0.833	3.779	18.0	12.60	50.8	55.92
Mx0	64.7	99.6	34.86	0.811	3.854	19.6	15.40	39.7	52.74
Mx1	67.3	100.8	33.50	0.779	4.086	20.0	15.40	46.8	53.08
Mx2	71.9	107.8	35.90	0.835	3.761	18.6	14.00	45.6	53.20
SEM	4.03	3.85	1.844	0.043	0.222	2.50	1.482	2.94	1.669

CV%	13.6	8.5	11.6	11.6	13.0	30.5	24.1	14.2	6.9
Probabilities									
Sex	0.285	0.645	0.285	0.285	0.373	0.307	0.066	0.158	0.052
DHQ	0.540	0.878	0.540	0.540	0.654	0.993	0.855	0.051	0.880
LIN[g]	0.449	0.720	0.449	0.449	0.461	0.937	0.594	0.025	0.705
Sex.DHQ	0.155	0.121	0.155	0.155	0.151	0.836	0.855	0.746	0.962
Interaction	0.838	0.090	0.838	0.838	0.743	0.752	0.689	0.781	0.915

Legend: a - FCR - Feed conversion ratio;
b - thickness of back fat in points X_1 and X_2;
c - X_3 (MLT) - Thickness of *m. Longissimus Thoracis* (MLT);
d - LM - lean meat percentage;
e - Level 0 - control group, without DHQ; level 1-100 g DHQ/kg feed; level 2-200 g DHQ/kg feed;
f - probability trough F criterion of Fisher;
g - LIN - linearity - linear raising of parameters at linear increase of amount of DHQ added to feed.

In this study, a trend for the effect of dihydroquercetin on the thickness of MLT, measured *in vivo* was found. Muscle thickness was higher in both DHQ-fed groups, by 12.05% and 13.69%, resp. than in the C group. It was very close to significance (P = 0.051), obeying a linear relationship (P = 0.025), and indicating that the addition of two successive levels of DHQ increased the value of the thickness of MLT in a gradient manner. This is an interesting result and has not been reported before. It indicating that, despite minimal differences in back fat thickness, and lean meat percentage, there is an effect of DHQ on MLT thickness. This important characteristic has a favourable effect on consumer expectations for a quality product. A similar result was found in our previous study with DHQ in fattening pigs, but with an inverse relationship regarding the increase in the amount of supplement in the diet. The dosage of 3.5 mg/kg live weight significantly increased MLT by 13.37% (P<0.05), and the dosage of 7.5 mg/kg by 6.63% (n.s.). In addition, dihydroquercetin may have a beneficial effect in the prevention of myopathic changes in the structure and proportion of muscle fibres in the MLT (Semenova et al., 2020). This result was found in a study of fattening pigs fed with a DHQ supplement at a dose of 40 mg/kg, given as an adaptogen to test the effect of a modelled technological stress on the state of muscle tissue.

The percentage of lean meat measured *in vivo* did not follow the trend found in MLT, i.e. no statistically significant differences were observed between the experimental and control groups. A trend was found for the effect of sex on the percentage of lean meat in female animals in the experiment (P = 0.052). Greater MLT thickness of 6.97% was observed in female animals. The percentage of lean meat was also higher in females compared to males - 2.78% in absolute terms. A statistically significant higher percentage of lean meat (*in vivo*) was found in our previous study using polyphenols (dry residue of distilled rose petal) in male entire pigs, and the differences with the castrated control group were significant (P = 0.006), but the trial ended at a higher live weight of the animals (Ivanova et al., 2021b).

The normal functioning of cells, organs and the body as a whole is maintained by homeostasis. The content of glucose in the blood is regulated by a multicomponent neuroendocrine complex, in which the sugar-lowering factor is insulin, and the sugar-raising factor is adrenaline, glucagon and glucocorticoids. In our study, as it is showed in Table 3, a dependence on DHQ intake was found to reduce blood glucose by 9.73% in the group of pigs receiving 100 mg/kg feed DHQ and by 11.09% in the group of pigs receiving 200 mg/kg feed DHQ (P<0.05). With this indicator, a linear dependence (P = 0.022) was established, which shows that the blood glucose content decreases linearly with an increase in the DHQ dose. The administration of dihydroquercetin in diets of the experimental groups played a role of protected liver function. Aa a result, a reduced glucose synthesis was observed within the physiological norm and an increased glucose content in the animals of the control group, demonstrating the hypoglycemic effect of DHQ. Our results were in line with those of Bule et al. (2019), who in a recent systematic review of the available literature in different animal species, using the method of meta-analyses, showed that quercetin reduced serum glucose levels at doses of 10, 25 and 50 mg/kg per kg body weight in fattening pigs. Studies by Fomichev et al. (2017) showed that supplementation of DHQ in diets of young pigs maintained blood glucose content within the physiological range, while in control pigs it was higher and may suggest increased function of the adrenal cortex. In relation to this, a presence of gluconeogenesis may appear. The trend we found also coincided with the action of flavonoids found in humans, which could regulate glucose metabolism, liver enzyme activity and lipid profile (Al-Ishaq et al., 2019), thus ameliorating the pathogenesis of diabetes and its complications. Vessal et al. (2003) also found that quercetin supplementation for two weeks reliably lowered blood glucose levels, increased the expression of genes involved in cell survival and proliferation in the liver, and enhanced serum insulin in STZ-induced diabetic mice.

Table 3. Biochemical indices in fattening pigs fed two levels of dihydroquercetin

Variables	Parameters									
	GLU	TRI	CHOL	HDL	LDL	ALT	ASAT	TotProt	CRE	UREA
Sex										
Female (F)	4.95	0.321	2.527	1.149	1.213	58.7	36.0	65.79	119.8	6.61
Male (M)	4.61	0.314	2.465	1.113	1.207	54.9	29.4	66.77	113.9	5.56
SEM	0.134	0.016	0.064	0.033	0.048	2.07	4.20	1.004	2.46	0.257
DHQ (dihydroquercetin)*										
Level 0	5.14[b]	0.334	2.400	1.070[a]	1.187	57.7	32.9	67.13	113.1	5.93
Level 1	4.64[a]	0.339	2.447	1.084[a]	1.188	53.5	29.6	64.71	121.1	5.90
Level 2	4.57[a]	0.280	2.640	1.239[b]	1.254	59.2	35.6	67.01	116.3	6.42
SEM	0.164	0.020	0.078	0.040	0.058	2.54	5.15	1.230	3.02	0.314
Fx0	5.30	0.318	2.340	1.066	1.144	60.8	41.8	67.32[ab]	115.8	6.32
Fx1	4.72	0.346	2.520	1.116	1.212	52.8	28.4	62.00[a]	122.2	6.68
Fx2	4.84	0.300	2.720	1.266	1.282	62.4	37.8	68.06[b]	121.4	6.82
Mx0	4.98	0.350	2.460	1.074	1.230	54.6	24.0	66.94[ab]	110.4	5.54
Mx1	4.56	0.333	2.375	1.052	1.165	54.2	30.8	67.42[b]	120.0	5.12
Mx2	4.30	0.260	2.560	1.212	1.226	56.0	33.4	65.96[ab]	111.2	6.02
SEM	0.232	0.028	0.110	0.057	0.082	3.59	7.28	1.739	4.27	0.444
CV%	10.8	19.9	9.9	11.3	15.2	14.1	49.8	5.9	8.2	16.3
Probabilities[c]										
Sex	0.085	0.759	0.500	0.441	0.934	0.215	0.278	0.497	0.102	0.008
DHQ	0.043	0.089	0.092	0.012	0.655	0.277	0.714	0.310	0.190	0.434
LIN[d]	0.022	0.069	0.040	0.007	0.425	0.124	0.714	0.946	0.096	0.281
Sex.DHQ	0.716	0.449	0.377	0.795	0.534	0.475	0.384	0.098	0.646	0.612
Interaction	0.508	0.848	0.519	0.686	0.668	0.227	0.295	0.037	0.456	0.327

Legend: * - 0 – control group, without DHQ; level 1 – 100 g DHQ/kg feed; level 2 – 200 g DHQ/kg feed;
Statistically significant differences are marked with different letters, as follows: a, b – probability at P < 0,05; c – probability trough F criterion of Fisher; d – LIN – linearity – linear raising of parameters at linear increase of amount of DHQ added to feed.

The addition of dihydroquercetin to the feed of fattening pigs in our experiment greatly had effect on their fat metabolism, with almost all indicators being affected in the direction of reduction. The results of the study (table 3) show that the intake of 200 mg of DHQ in fattening pigs had a significant effect on the content of triglycerides in the blood. A trend was found for their reduction by 16.17% (P<0.01), and it was more pronounced in male animals, which values were lowered by 25.71% (P<0.01). Total cholesterol values, however, showed a linear increase (P=0.040) with increasing DHQ dose, with a more pronounced effect at the higher DHQ dose (200 mg/kg feed). The blood cholesterol content of all pigs consuming the supplement at this dose was higher than that of control pigs by 9.09% (n.s.). The effect of gender was shown, with the difference being higher in females (13.97%) compared to males (3.90%). Interestingly, the trend shown for the recorded increase in blood cholesterol content was at the expense of the so-called "good" or high-density cholesterol (HDL). This increase of 13.64% in DHQ2 animals showed high probability (P = 0.012) and high linear dependence (P = 0.007). This means that the gradient levels of dihydroquercetin raised the content of "good" cholesterol. Differences in this indicator by gender between the treated and control groups were in favour of female animals as well (15.80% vs. 11.39% in males).

It is evident from the literature that very few studies have been conducted regarding the effects of quercetin and especially dihydroquercetin on lipid metabolism in pigs. Furthermore, the interpretation of the results of the present study suggests that dihydroquercetin lowers blood lipids to levels similar to the effects of other flavonoids, and that the results for blood serum cholesterol content are different from organ cholesterol content. A much more detailed study showed that quercetin reduced triglyceride content via the PPAR signalling pathway in primary hepatocytes of broiler chickens (Wang et al, 2019). Moreover, flavonoids from sea buckthorn fruit had a quadratic effect on the content of triglycerides and VLDL in the liver of broiler chickens (Han et al., 2009). In a study of Tang et al. (2013), quercetin treatment significantly reduced cholesterol content in the liver, heart, kidney and small intestine of rats. Quesada et al. (2009) reported that effect of treatment with grape seed procyanidin extract caused slightly reduction in triglyceride and cholesterol content in the liver of rats. Zhai et al. (2016) found that quercetin reduced serum triglyceride content in tilapia (freshwater fish), which may be useful to avoid pathological changes in fatty liver. Kuipers et al. (2018) also reported that quercetin reduced plasma triglyceride level in 9-week-old mice. Additionally, a flavonoid from hawthorn leaf extract significantly reduced serum cholesterol, triglycerides, and very high-density lipoprotein (VHDL) cholesterol levels in mice.

In our study, no statistically significant differences were found between the control and experimental groups, as well as between the two groups with different levels of DHQ supplementation, regarding the values of the liver enzymes AST and ALT (table 3), which give an idea of the production of enzymes in organism. Under physiological conditions, these enzymes are present in small amounts in the peripheral blood. ALT is a specific marker of the functional state of the liver, and an increase in AST activity is characteristic of disorders of the functions of the cardiovascular system. In our study, dihydroquercetin did not affect the levels of these enzymes in the blood, but in another study with pigs, it was found that after administration of the larch bioflavonoid complex, the activity of alanine aminotransferase decreased by 12.8%, of aspartate aminotransferase by 23, 5% (Fomichev et al., 2017).

A statistically significant difference between the groups as well as an interaction (P=0.037) was found for total blood protein content, with female animals in DHQ1 group having a lower total protein by 8.90% of the other experimental group DHQ2, but both groups had no statistically significant differences with the control group. No differences were found due to the administration of dihydroquercetin in the indicators of protein metabolism, creatinine and urea content in the blood, as well. According to the last indicator, a statistically significant difference of 15.88% was found between male and female animals in favour of females (P=0.008), but such a difference is a gender feature that is also characteristic of humans (Liu et al., 2021).

During the entire study, for the entire experimental period of 43 days, no cases of animal disease were recorded, i.e. Throughout the study, clinically healthy animals were used, which is evident in the values of the biochemical indicators, all of them being within the reference limits. This may be the reason why the activity-stimulating action of dihydroquercetin in the direction of stabilization and improvement of the work of all organs and systems was not manifested. The biologically active substance DHQ was used in animal husbandry, especially when the farming of animals was carried out in areas contaminated with anthropogenic heavy metals (Pb, Cd, As, Hg and others) and radionuclides (90Sr, 137Cs) or they were exposed to pollution from industrial enterprises from the chemical, metallurgical, petrochemical and other industries (Fomichev et al., 2016).

CONCLUSIONS

Administration of dihydroquercetin did not affect parameters of pig performance in fattening period from 66 to 100 kg live weight.

The addition of two consecutive levels of DHQ increased MLT, measured *in vivo*, linearly (P=0.025).

Dihydroquercetin had a positive effect on carbohydrate metabolism in fattening pigs in the direction of reducing blood glucose content, with linear reliability at both levels used 100 mg/kg and 200 mg/kg feed (P<0.05).

The intake of DHQ affected the fat metabolism of pigs. The addition of DHQ at a dose of 200 mg/kg feed to the diets of fattening pigs showed a trend to reduce the content of triglycerides and linearly increase total cholesterol (P<0.05), and this increase was at the expense of high-density cholesterol HDL (P<0.05).

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EFFECT OF FEEDING WITH FROZEN AND DRIED POLLEN ON THE DEVELOPMENT OF BEE COLONIES

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Abstract

This study aimed to examine the impact of feeding with frozen and dried pollen on the development of bee colonies (Apis mellifera L.). The study monitored changes in the strength of the bee colonies (SBC), the amount of sealed worker bee brood (SWBB), and food reserves (honey and pollen) before and after feeding during the Autumn and Spring periods. The experiment was conducted with one control group (CG) and two experimental groups - two bee colonies fed with frozen pollen (GFFP) and two bee colonies fed with dried pollen (GFDP). The results showed that during the Spring period, the SBC fed with frozen pollen was 36.52% higher than the SBC of the CG. For the same period, a higher value of the amount of pollen and the amount of SWBB was also observed in the GFFP compared to its value in the CG. In the Autumn period, the amount of brood in GFDP was 51.20% more than in the CG. In the GFFP, a greater amount of brood was also found compared to the CG, with the difference being 48.30%.

Key words: *Apis mellifera L., development of bee colonies, feeding, frozen and dried pollen.*

INTRODUCTION

The development of bee colonies throughout the year is a dynamic process associated with adaptation to constantly changing environmental factors (Cebotari & Buzu, 2023). The number and functions of the separate individuals, as well as the relationships between them, are constantly changing and in this way, the maximum utilization of the available honey-bearing vegetation is reached.

According to Nedyalkov et al. (1991), the year-round life of the colony is divided into two periods - a period of active work and a period of relative rest, associated with the seasons spring - summer and autumn - winter, respectively. The period of active work begins with the laying of the first eggs by the queen bee in January - February. Bee activity increases when they begin to feed the first larvae in the hive. With the increase in the proportion of flowering vegetation in the spring, the brood in the bee colonies gradually increases and reaches its amaximum in May-June, when in one day the queen bee lays 1500-2000 eggs. At the beginning of the main pasture, the colony reaches the highest point in its development during the year. Collecting pollen in autumn is very important for replenishing the reserves in the bees' fat body. This, in turn, ensures proper wintering and early brood rearing in the spring (Bizhev et al., 2003).

In periods when brood is being raised in the bee nests, the “nurse” bees need a large amount of protein, which they obtain from the pollen entering the hive. It is carried by the “forager” bees. They use the secretion of their salivary glands to shape the collected pollen from plants into pollen grains, which they place in baskets made of hairs on the third pair of legs (Garbuzov & Ratnieks, 2014). When the “forager” bees arrive at the hive, they place the pollen grains in the comb cells, compacting them, replenishing the cells with honey and finally sealing them with wax caps (Nicolson et al., 2018).

After the pollen cells in the bee comb are sealed, lactic acid fermentation takes place with the participation of strains of lactic acid bacteria (Nepi et al., 2018). Thanks to the fermentation, acidity increases and thus prevents unwanted processes in the pollen, and also improves its nutritional value. After these changes in the pollen, it becomes “bee bread”, which is the main source of protein for bees. The availability of pollen in bee colonies is particularly important in the production of royal jelly and the rearing of larvae and young worker bees (Abou-Shaara, 2014).

The development of modern agriculture has largely led to a change in the natural habitats of pollinators. This also limits the diversity of floral resources for honey bees (Biesmeijer et al., 2006). Furthermore, seeding large areas with agricultural crops of the same species reduces the diversity of flowering plant species within the flight radius of bees around hives (Grundel et al., 2010; Rands & Whitney, 2010). Mass flowering monocultures provide temporary availability of nectar and pollen for bees (Todd et al., 2016). For these reasons, the loss of biodiversity of floral resources can lead to suboptimal bee nutrition, which in turn leads to reduced bee immunity and poor health (Dolezal et al., 2019; De Grandi-Hoffman et al., 2010).

The natural foods for bees (nectar, manna, honey, pollen, royal jelly) contain all the nutrients necessary for the normal development of bee individuals. Regardless, in beekeeping practice, supplemental feeding of bee colonies is practiced, aiming to compensate for the lack of food coming into the bee colonies from nature. Supplemental feeding of bee colonies is also relevant in connection with global warming and climate change. The presence of long periods of drought and lack of nectar and pollen in nature leads to stress in the development of the colonies and to their death.

Regarding the proper breeding and development of bee colonies, we consider it reasonable to study the effect of supplemental feeding of bees with dried and freshly collected frozen pollen.

MATERIALS AND METHODS

The survey was conducted in the fall of 2022 and the spring of 2023 at the Educational Experimental Base of the Beekeeping section at the Faculty of Agriculture of Trakia University - Stara Zagora. Feeding of bee colonies of the local honey bee (*Apis mellifera* L.) settled in Dadant-Blatt 10-frame hives was carried out. The colonies were previously equalized by the age of the queen bees, strength, amount of brood, and food reserves. Two experimental and one control groups were formed with two bee colonies each, as follows:

- **1st Experimental group (GFDP)** – six feedings at seven days' intervals with 160 g polyfloral bee honey mixed with 40 g of dried pollen;
- **2nd Experimental group (GFFP)** – six feedings at seven days' intervals with 160 g polyfloral bee honey mixed with 40 g of frozen fresh pollen;
- **Control group (CG)** – six feedings at seven days' intervals with 200 g polyfloral bee honey;

The development of the bee colonies from the experimental and control groups was monitored in terms of the following indicators - strength, amount of sealed worker brood and food reserves (honey and pollen) in the autumn and spring periods, before and after feeding. Classic methods established in beekeeping were used to determine the indicated parameters.

1. Determining the strength of bee colonies (SBC)

To determine the strength of the bee colonies, it is taken into account that in the Dadant-Blatt hive, a densely occupied inter-frame space accommodates about 0.250 kg of bees. The inter-frame spaces occupied with bees are counted and their number is multiplied by 0.250 kg. Since the weight of one worker bee is approximately 100 mg, it is assumed that there are 10,000 bees in 1 kg.

2. Determining the amount of sealed worker brood (SWBB)

When determining the amount of sealed worker brood, a measuring frame with 5/5 cm squares was used, which was placed on the brood comb (Figure 1). One square of the measuring frame has an area of 25 cm^2. One square centimeter accommodates 4 worker cells, and 25 cm^2 – 100 cells (Nenchev & Zhelyazkova, 2010).

From the specified amount of brood, the bees will hatch in a period of 12 days, because the pupa stage (sealed brood) lasts 12 days. For this reason, the brood measurement is carried out over 12-day periods.

3. Determining the amount of sealed honey and pollen in the combs

- amount of honey – only the sealed honey is determined

To determine the area of sealed honey, a measuring frame with 5/5 cm squares is placed over the measured honeycomb (Figure 2). The area of one square in the measuring frame is 25 cm^2.

Figure 1. Determining the area of sealed brood with a measuring frame (own source)

Figure 2. Determining the area of sealed honey with a measuring frame (own source)

When determining the amount of honey in kilograms, it is assumed that there is 0.350 kg of honey on an area of 1 dm^2 (100 cm^2) of the comb filled on both sides, 0.175 kg on one side, respectively (Nenchev & Zhelyazkova, 2010). After counting the filled squares, the area of the sealed honey is determined by the following formula (Lazarov & Dineva, 2022):

$S_{hf} = \mathrm{n}.25, \mathrm{cm}^2$

where n is the number of squares filled with honey.

The total amount of honey on one side of the comb is calculated by the following formula:

$$Q_{hf} = \frac{S_{hf}.0{,}175}{100}, kg$$

where: Qhf – amount of honey, kg;
Shf – area of the sealed honey, cm^2

- amount of pollen – determined with a measuring frame with 5/5 cm squares. Within one square of the measuring frame, there are 25 cm^2. The measuring frame is placed on the pollen combs counting the squares filled with pollen.

Statistical software IBM SPSS Statistics 26.0 (NY, USA) was used for data processing. Univariate ANOVA analysis was used to study the effect of supplemental feeding of bee colonies with frozen and dried pollen on the development of bee colonies in the autumn and spring periods. Significant differences between the groups at $p < 0.05$ were calculated by the Post Hoc procedure with the Scheffe test. Levene's test was applied to verify the data variations.

RESULTS AND DISCUSSIONS

1. Bee colony strength

Figure 3 shows the results from the Univariate ANOVA analysis regarding the bee colony strength for the periods after spring and autumn supplemental feeding with dried and frozen fresh pollen.

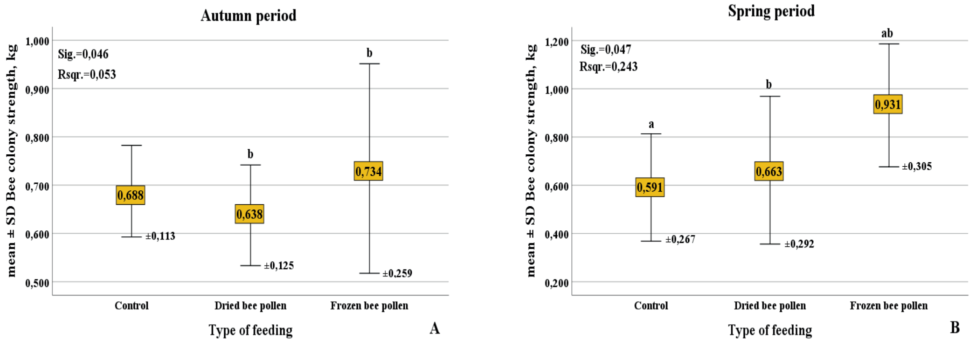

Figure 3. Strength of bee colonies, kg

In the autumn period after feeding the bee colonies, close average values of the strength of the bee colonies were found in the experimental and control groups: GFDP - 0.638±0.113 kg, GFFP - 0.734±0.125 kg and CG - 0.688±0.113 kg, respectively. A significant difference was reported between the two experimental groups (Figure 3A).

A significant difference in the strength of bee colonies fed with frozen pollen and the CG was observed during the spring period (Figure 3B). The strength of bee colonies from this group was 0.931±0.305 kg, and for colonies from the CG, it was 0.591±0.267 kg. The indicated difference is 36.52%, which is indicative of the effect of feeding with frozen pollen. Probably, with this way of storing the pollen, its qualities are preserved to the greatest extent compared to drying it. De Grandi-Hoffman et al. (2016) found that feeding bee colonies with floral pollen before wintering and in early spring positively influences their wintering ability and supports the development of bee colonies during the spring period. The authors establish that bees fed with floral pollen were stronger, healthier, and more active. In colonies fed with dried pollen during this period of the year, the difference compared to the control group is insignificant - 11%. Similar to the autumn period, a statistically significant difference between the two experimental groups fed with frozen and dried pollen was recorded in the spring. The coefficients of determination R^2 (0.053; 0.243) for both periods show that from 5.3% to 24.5% of the variations in the SBC are due to the type of feeding. This again confirms the significance of the type of pollen used for feeding to the strength of bee colonies.

2. Amount of honey

Figure 4 presents the results from the Univariate ANOVA analysis regarding the presence of sealed honey in bee nests for the period after spring and autumn feeding with dried and frozen fresh pollen.

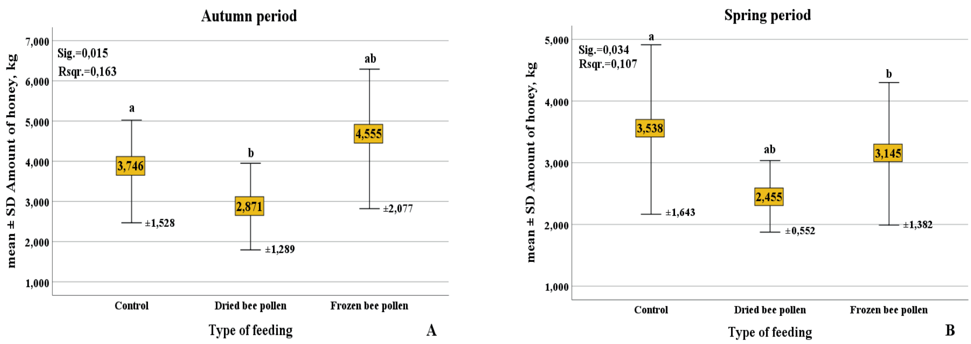

Figure 4. Amount of honey in bee colonies, kg

In the autumn period, it was found that in the GFFP, the average value of the amount of honey was 4.555±2.077 kg, which is 17.76% higher compared to the value of this parameter in the colonies from the CG. A probable reason for this is that during this period the strength of colonies (number of bees) from this group was higher (GFFP - 0.734±0.125 kg) and CG (0.688±0.113 kg), respectively (Fig. 3A). The analysis of the results for the autumn period illustrated on Figure 4A shows that the average value of the amount of honey of the GFDP (2.871±1.289 kg) was lower than that of the CG (3.746±1.528 kg). It can be assumed that this is also due to the lesser strength of the colonies from the experimental group fed with dried pollen compared to the strength of the families from the CG (Figure 3A). A significant difference was found regarding the amount of honey in bee nests between the two experimental groups.

After the spring feeding, statistically significant differences were recorded between the average values of the amounts of honey obtained from the CG (3.538±1.643 kg) and the GFDP, as well as between the two experimental groups - GFDP (2.455±0.552 kg) and GFFP (3.145 ±1.382 kg). The coefficients of determination R^2 (0.107 and 0.163) for the two periods show that from 10.7% to 16.3% of the variations in the amounts of honey produced by the bee colonies are due to the type of their supplemental feeding, which reflects on their strength.

3. Surface area bee pollen

Figure 5 presents the results of the Univariate ANOVA analysis regarding the area of bee pollen in the combs stored by the bee colonies for the periods after the spring and autumn feeding with dried and frozen fresh pollen.

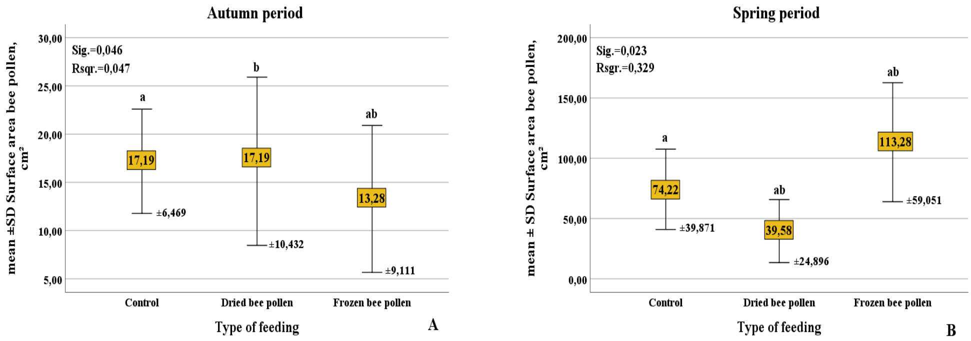

Figure 5. Bee pollen area, cm^2

When analyzing the results for pollen content in bee colonies after feeding, it was found that in the autumn period, the value of this parameter for the GFDP was the same as for the CG (17.19 cm^2), (Figure 5A). The value of the indicator in the GFFP pollen was even lower (13.28 cm^2). A possible reason for this is that bee colonies do not store the extra pollen supplied by the experiment.

During the spring period (Figure 5B), significantly larger areas of pollen in combs were observed in the GFFP than in the CG, 113.28±59.051 cm^2 and 74.22±39.58 cm^2, respectively. This difference is meaningful. It is possible that the bees mix and process the pollen that is additionally provided to them in the experiment with that which they bring into the hives from nature and store it in the combs. The presence of pollen in the bee colonies during this period is particularly important in the production of royal jelly and the rearing of the larvae and young worker bees (Abou-Shaara, 2014). Significant differences were found in the pollen content of bee colonies fed with dried and fresh frozen pollen, both in autumn and in spring. The coefficients of determination R^2 (0.047 and 0.329) for the two periods show that from 4.7% to 32.9% of the variations in pollen content of the combs are due to the type of feeding of the bee colonies.

4. Surface area capped brood

Figure 6 presents the results of Univariate ANOVA analysis on the area of sealed worker brood for the periods following spring and autumn feeding with dried and frozen fresh pollen.

Regarding the influence of supplemental feeding of the bee families and brood keeping in the autumn period, it was observed that in the colonies receiving dried pollen, the amount of brood was 390.63±337.781 cm^2, which is 51.20% more than in the CG (190.630±252.446 cm^2), (Figure 6). This is a significant difference, and bearing in mind that for the successful wintering of bee colonies, it is necessary to provide them with a large number of worker bees to hatch in the autumn, it is not to be neglected at all. Some authors establish a relationship between the nutritional value of pollen and the development, reproduction, and productivity of bee colonies (Radev et al., 2014). In the GFFP, a greater amount of brood was also found compared to the CG, the difference being 48.30% (Figure 6A). These results show the beneficial effect of supplemental feeding of bee colonies during the autumn period, when in nature the availability of pollen is limited. A similar statement is shared by other authors (Bizhev et al., 2003).

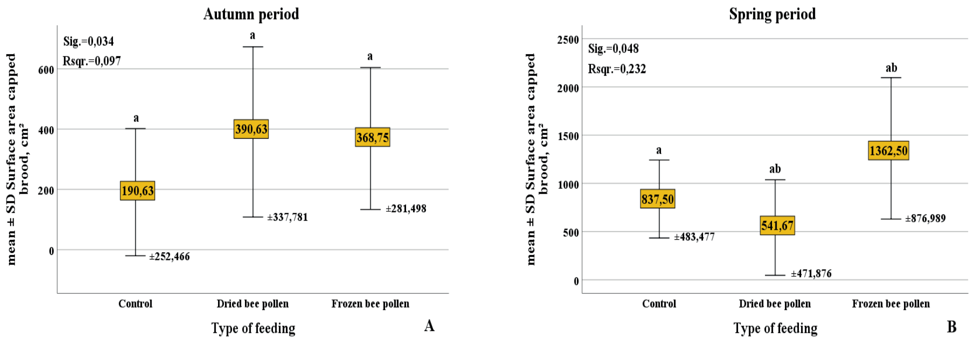

Figure 6. Area of capped worker brood, cm^2

Seasonal and climatic changes throughout the year lead to a significant decrease in resources from nectar-producing plants in nature (Şahin et al., 2015). When natural nectar-producing vegetation is insufficient, the egg-laying rate of the queen bee decreases, which in turn leads to a reduction in the quantity of brood and the number of bees in the bee colony.

Figure 6B shows that during the spring period feeding colonies with dried pollen does not lead to an increase in the amount of brood. In this group, its amount is less than in the CG. Analysing the results for this indicator in the GFFP during the spring period, it can be seen that the amount of brood is 38.58% higher than its level in the CG, and this difference is statistically significant (Figure 6B).

The coefficients of determination R^2 (0.097 and 0.232) for the two periods show that from 9.7% to 23.2% of the variations in the amount of brood are due to the type of feeding of the bee colonies.

CONCLUSIONS

During the spring period, the strength of bee colonies from the group fed with frozen pollen was 0.931±0.305 kg, and for colonies from the control group, it was 0.591±0.267 kg. The indicated difference is 36.52%, which is indicative of the effect of feeding with frozen pollen during this period of the year. In the colonies fed with dried pollen during the same period of the year, the difference compared to the control group is insignificant - 11%.

During the spring period, significantly larger areas of pollen in the combs were observed in the group fed with frozen pollen than in the control group, 113.28±59.051 cm^2 and 74.22±39.58 cm^2, respectively. This difference is significant, and it is likely that the bees mix and process the pollen that is additionally provided to them in the experiment with the one they bring into the hives from nature and store it in the combs.

During the autumn period, it was observed that in the colonies receiving dried pollen, the amount of brood was 390.63±337.781 cm^2, which is 51.20% more than in the control group (190.630±252.446 cm^2). In the group fed with frozen pollen for this period, a greater amount of brood was also found compared to the control group, the difference being 48.30%. The results for this indicator show that the amount of brood in the group fed with frozen pollen in the spring period is 38.58% higher than its value in the control group.

For all studied parameters, significant differences were found between the two experimental groups, both in the autumn and in the spring period, which confirms the authors' thesis that the type of pollen used for supplemental feeding has a significant impact on the development of bee colonies.

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EFFECTS OF DIETARY PROSO MILLET ON PERFORMANCE, PROTEIN PROFILE, NITROGEN BALANCE, AND GREENHOUSE GAS EMISSIONS OF GROWING PIGS

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Abstract

This study aims to evaluate the impact of 25% proso millet on growth performance, plasma protein profile, and nitrogen balance and to predict the releases of some greenhouse gas emissions (GES) from enteric fermentation and in the growing pig manure. During 21 days, two groups of 10 castrated male Topigs pigs with the same weight (30.48 ± 0.26 kg) and age (81 ± 3d) were fed: control (corn-triticale-soybean meal, C) or experimental (corn-proso millet-soybean meal, E). The animals were kept in metabolic cages. The GES calculation model was based on the IPCC (2016) approach, incorporating experimental parameters and prediction equations. During the analyzed period, the dietary treatment, increased (P<0.05) the growth parameters while the plasma protein profile was not significantly affected. In the E group, total nitrogen excretion (4.3%, P<0.05), and nitrogen digestibility (6.1%, P<0.05) increased while enteric CH_4, g Eq.CO_2 decreased (14.8%, P<0.05) vs. the C group.

Key words: *growing pigs, greenhouse gas emission, nitrogen balance, plasma protein profile, proso millet.*

INTRODUCTION

Commonly cultivated in the world, proso millet (*Panicum miliaceum* L.) is an important energo-proteic source for humans and animals (Baltensperger, 2002; Habiyaremye et al., 2017). It is also rich in minerals, vitamins, and different micronutrients. Thus, millet grains have a content of 9-11% crude protein, 2-4% crude fat, 15-20% fibre (Dayakar et al., 2017; Lefter et al., 2020). It has progressively been substituted by wheat and corn in the Western diet, although it continues to be extensively cultivated in India, Russia, the Middle East, Turkey, and Romania. Specifically, proso millet is a summer annual grass belonging to the Poaceae family, reaching maturity in 60-100 days. According to Joshi et al. (2023) and Ramesh et al. (2024), proso millet proved to be a resilient crop in this changing climate. The unique characteristics of this plant, such as the strength of its root structure, enable it to thrive in arid regions and on non-irrigated land with only 200-500 mm of annual rainfall, as well as in flooded areas (Changmei & Dorothy, 2014; Habiyaremye et al., 2017; Bhat et al., 2019). Recently, the interest in the potential of millets has gained importance as C_4 plants, particularly for the ability to efficiently utilize atmospheric CO_2 and due to other agronomic traits (Saxena et al., 2018; Kheya et al., 2023; Ţîţei V., 2023). The pig meat is an important protein source for human consumption and the amount of meat produced is related highly to the quality of feed provided. However, the pollution generated by pig farms can come from the decomposition of manure, which leads to environmental problems that affect the atmosphere and human health. According to data provided by the Organization for Economic Cooperation and Development (OECD), the environmental consequences caused by this sector are at the centre of concerns, especially regarding the management of pig manure and related water and air pollution. The European Parliament Resolution on the strategy for a long-term reduction of greenhouse gas (GES) emissions, following the Paris Agreement 2019/2582 (RSP) emphasizes that: net emissions will have to be reduced to values close to zero in all sectors of the economy, which requires common efforts across all sectors; pathways for climate neutrality must be developed for all sectors; *"the polluter pays principle applies"*.

The GESs are gaseous compounds that trap heat or long-wave radiation from the atmosphere. The greenhouse effect represents the natural process of heating the earth's surface. The main sources and types of GESs from the livestock sector are: methane (CH_4) which represents 25% of the emissions, CO_2 around 32% and nitrous oxide (N_2O) around 31%. These gases are usually converted to CO_2 equivalent (CO_2 eq.) units as an expression of global warming potential.

Nitrogen (N) is a component of feed protein, participates in numerous metabolic processes, and is one of the most expensive nutrients in pig's diet. Thus, one important way to minimize nitrogen excretion (NE) is by feeding (Millet et al., 2018; Wang et al., 2018a).

Presently, the emission of N_2O from the livestock sector has increased significantly. N_2O is a greenhouse gas with a global warming potential 298 times that of the reference CO_2 (IPCC, 2006). According to Dourmad et al. (2017) and Millet et al. (2018) only 32-46% of ingested N is retained by pigs.

Methane is a volatile organic compound resulting from the digestion processes that take place in the digestive tract (enteric) and following the fermentation processes in the excreta (Liu et al., 2022). The global warming potential is 25 times that of CO_2.

In pig farms, CO_2 emissions come from exhalation and are released through manure (Philippe & Nicks, 2014), although the latter represent negligible amounts. CO_2 is taken up by plants throughout the photosynthesis process. While CO_2 in manure comes from urea hydrolysed into NH_3 and CO_2, from anaerobic fermentation processes of organic matter resulting in intermediate products volatile fatty acids, CH_4 and CO_2 as well as from aerobic degradation of organic matter (Philippe & Nicks, 2014).

Recent studies have highlighted some possibilities to reduce nitrogen emissions from farms such as nutritional strategy (Mihăilă et al., 2023; Popa et al., 2022) or the adoption of the Internet of Things devices applied on farm-based models (Popa et al., 2021).

This study aims to evaluate the impact of 25% proso millet on growth performance, plasma protein profile, and nitrogen balance (NB) and to predict the releases of some GES from enteric fermentation and in the growing pig manure.

MATERIALS AND METHODS

Animals and layout of the investigation

The balance trial was authorized by the Ethics Committee of the National Research Development Institute for Animal Biology and Nutrition Balotesti, Romania, following the European legislation (Directive 2010/63/EU) and the Romanian Law no. 199/2018 for animal trials.

Ten healthy growing TOPIGS pigs [females Large White × Hybrid (Large White × Pietrain) and boar Talent, particularly Duroc] with an average live weight of 30.24 ± 0.26 kg were assigned to one of two dietary treatments with five castrated males per group.

The pigs were fed with both a standard diet (C) and an experimental diet (E), where the C diet's triticale grain was totally substituted with 250 g/kg of millet grain in the E diet. Table 1 shows that the formulated diets were isoenergetic, isonitrogenous, and with similar content in essential amino acids (lysine, methionine, and cysteine). Throughout the 21-day experiment, all pigs were provided free access to fresh drinking water and feed. The tested diets were provided to the pigs in pelleted form.

Table 1. Nutritional value and feed composition of the diets used in the experiments during phase

Items (g x kg^{-1})	C	E
Corn grain	434.0	431.8
Triticale grain	250.0	0
Millet grain	0	250.0
Rice meal	100.0	100.0
Soybean meal	180.0	180.0
Soybean oil	5.0	5.0
DL-Methionine	0	0.6
L-Lysine	1.7	2.3
Calcium carbonate	15.7	15.2
Monocalcium phosphate	1.5	3.0
Salt	1.0	1.0

Items (g x kg^{-1})	C	E
Choline premix	1.0	1.0
Vitamin-mineral-premix P3+4[a]	10.0	10.0
Phytase (500 FTU kg·feed^{-1})	0.1	0.1
Nutritional value of diets		
Dry matter	872.6	880.1
Crude protein	145.9	149.9
Digestible protein	127.4	126.9
Crude fat	27.7	35.0
Cellulose	44.9	41.5
Crude fiber	27.9	28.8
Hemicellulose	72.0	76.0
NDF	107.4	113.1
ADF	35.4	37.1
Starch	509.9	512.0
Lysine	9.7	9.7
Digestible lysine	7.7	7.9
Methionine + cysteine	6.3	6.3
Digestible methionine + cysteine	5.0	5.1
Calcium	8.0	8.0
Total phosphorus	5.9	5.9
Digestible phosphorus	2.1	2.2
Crude ash	48.0	50.5
Metabolizable energy (EM, MJ/kg)[b]	13.3	13.4
Net energy (NE, Mj/kg)[c]	10.2	10.3

[a]Supplies per kg of diet: vitamin A-6000 IU; vitamin D_3-800 IU; vitamin E-20 IU; vitamin K_3-1 mg; vitamin B_1-1 mg; vitamin B_2-3.04 mg; vitamin B_3-10 mg; vitamin B_5-6.3 mg; vitamin B_6-1.5 mg; vitamin B_7-0.03 mg; vitamin B_9-0.3 mg; vitamin B_{12}-0.02 mg; Mn-30 mg; Fe-80 mg; Cu-25 mg; Zn-100 mg; I-0.22 mg; Se-0.22 mg; Co-0.3 mg; antioxidant-60; NDF, neutral-detergent-fiber; ADF, acid-detergent-fiber; ME and NE[b,c], calculated values using regression equations.

Plasma protein metabolites

Blood samples were collected in heparinized vacutainers by venipuncture (jugular vein), under aseptic conditions from all of the pigs (n=5/group), and the determinations were done in triplicate. Plasma concentrations of total protein (TP), bilirubin (Bil), albumin (Alb), creatinine (Cre), blood urea nitrogen (BUN), and uric acid (UA) were determined using the Spotchem EZ SP-4430 analyzer (Arkray, Japan) and specific reagent kits.

Balance trial

Each pig was kept in individual metabolism cages made from steel. The metabolic cages were set in a room equipped with an environmental computer-controlled system located at the INCDBNA-IBNA Experimental Biobase. The first week served as an adaptation period, succeeded by two balance periods. In this second period, each day the fresh urine and faeces were collected separately, weighed and 10% kept in the freezer at −18°C. The H_2SO_4 at 25% concentration was used in each urine container for acidification and proper presser-vation. At the end of the balance period, a representative sample was obtained from each animal and subjected to additional analysis. The N content in the samples of urine and faeces was determined using a semiautomatic Kjeltec Auto 1030 Analyzer (Hillerod, Denmark) according to the protocol described by Untea et al. (2012).

Kleiber ratio and relative growth rate calculation

The Kleiber ratio (KR) and the relative growth rate (RGR) were calculated according to the equations developed by Diaz et al. (2017). The KR was calculated according to the average daily gain (ADG) and the metabolic body weight (BW^0.75). For the RGR calculation, the following equation was used: 100 × [($\log_{10}$ final BW) − ($\log_{10}$ initial BW)]/(animal age at the end of the experiment – animal age at the start of the experiment). The voluntary daily feed intake (VFI) was the amount of feed ingested by pigs/day.

N balance parameters

The N balance parameters were determined using input data nitrogen intake (NI, as dry matter (DM) basis) and excretion. Based on previously developed equations (Habeanu et al., 2021a), the total nitrogen excreted (TNE), nitrogen retained (NR), coefficient of total tract apparent digestibility (CTTAD), coefficient of metabolizability (CAM), and net protein utilization (NPU) were calculated. Thus, TNE was rated as the difference between NI and nitrogen excreted (NE). The NPU was rated as the ratio of NR to NI. CTTAD and CAM were calculated using the equations: CAM = [(NI – NE − UNE)/NI]; CTTAD = [(NI – E)/NI], where: UNE means urinary nitrogen eliminated.

N_2O, CO_2 exhaled prediction and e-CH_4 and CH_4 manure production

The prediction of the exhaled nitrogen protoxide (N_2O), carbon dioxide (CO_2), enteric methane (e-CH_4), and CH_4 in the pig manure were calculated by corroborating our experi-mental data, literature prediction equations adapted by Habeanu et al. (2019a) and the calculation model based on the methodology proposed by IPCC (2006).

For the prediction of e-CH_4 expressed as eq. CO_2, was used the equation proposed by Philippe & Nicks (2014): e-CH_4=0.012 x dRes x DM (kg per day), where dRes (g per day) refers to the digestible residue. Exhaled CO_2 production was estimated using the equation developed by Rigolot et al. (2010). This equation used heat production (HP) estimated based on 0.163 l/h CO_2 per Watt of heat which was applied a correction [CO_2 density (22.4 l/mol), and on the molecular mass (44 g/mol)]. The N_2O emission was calculated by the amount of NE and the conversion factor (0.2) proposed by the IPCC, 2006, cited by Philippe & Nick. (2014).

Statistical calculations

The experimental data are reported as means and SEM (standard error of the mean). The Shapiro-Wilk test was applied for the distribution data model. The experimental data were managed using the SPSS V.20 (2011). $P<0.05$ was used to determine whether or not there were significant differences between values.

RESULTS AND DISCUSSIONS

Growth parameters

As shown in Table 2, the final body weight (BW) and was 5.8% higher in the E group ($p<0.0001$) comparing the C group.

Table 2. Growth parameters and fibre average daily intake of growing pigs

Items	C	E	SEM	*P-value*
Initial BW, kg	30.43	31.05	0.40	*0.311*
Final BW, kg	52.60	55.64	2.05	*<0.0001*
Final $MBW^{0.75}$	17.23	18.19	0.51	*<0.0001*
ADG, kg	1.10	1.26	0.14	*0.046*
KR	7.33	7.92	0.41	*0.010*
RGR	1.31	1.38	0.37	*0.001*
VFI, kg/day	2.71	2.78	0.03	*0.586*
DM VFI, kg/day	2.37	2.45	0.03	*0.588*
Gain-to-feed ratio, kg	0.41	0.47	0.04	*0.263*
ME consumption, MJ/kg $MBW^{0.75}$	9.99	9.82	0.04	*0.351*
Fibre average daily intake (g/head/day)				
Total fibre	109.1	103.2	4.00	*0.522*
Hemicellulose	217.4	227.1	6.23	*0.588*
Cellulose	117.3	109.2	5.63	*0.161*
NDF	282.1	296.2	9.67	*0.251*
ADF	107.1	111.1	2.73	*0.186*

BW, body weight; $MBW^{0.75}$, metabolic BW; ADG, average daily gain; KR, Kleiber ratio; RGR, relative growth rate; VFI, voluntary daily feed intakes; DM VFI dry matter voluntary daily feed intakes; ME, metabolizable energy; NDF, neutral-detergent-fiber; ADF, acid-detergent-fiber; SEM, standard error of the mean; $P<0.05$, significant difference between means; $P<0.01$, distinctly significant difference between means; $P\leq 0.001$, highly significant difference between means.

Also, for the average daily gain (ADG), KR, and RGR parameters, significant improvement (14.5%, 8.1%, and 5.3%) was observed. Indicators: VFI, VFI as DM, gain-to-feed ratio, and metabolizable energy (ME) consumption showed no significant differences ($p>0.05$) among groups.
The findings of our study are in concordance with other research (Bastos et al., 2006; Berglund, 2007; Nguyen, 2022) indicating that including different varieties of millet grain to growing pigs diets, even at 50% or more, did not negatively impact performance.
As well, the substitution of corn with millet between 5% and 75% in broiler diet resulted in similar (Davis et al., 2003; Hidalgo et al., 2004) or improved performance (Baurhoo et al., 2011) compared with corn diets.
Instead, Hidalgo et al. (2004) and Cisse et al. (2016) showed that the inclusion of millet in broiler diets did not influence body weight gain or feed efficiency compared to the corn diet.

Plasma protein metabolites

Table 3 shows the plasma protein metabolites, valuable health status indicators, and nitrogen metabolism. In our experiment, no significant differences were noticed in response to the dietary changes. Additionally, all analyzed blood parameters fell within the standard range for this pig category, implying that the diets had no adverse effects. Lefter et al. (2020a; 2020b; 2021) obtained comparable results when substituting 25% of triticale with millet in the diets of weaning and growing pigs. The lack of significant variations in blood protein levels, as observed in our current study when incorporating millet into the experimental diet, suggests a well-balanced composition of amino acids in the feed and the efficient nutrient utilization of pigs.

Table 3. Plasma protein metabolites of growing pigs

Items	Reference values*	C	E	SEM	*P-value*
TP (g/dL)	5.8 - 8.3	5.22	5.31	0.02	*0.422*
T-Bil (mg/dL)	0 - 0.5	0.20	0.24	0.01	*0.450*
Alb (g/dL)	2.3 - 4.0	2.51	2.54	0.01	*0.370*
Cre (mg/dL)	0.8 - 2.3	1.11	1.10	0.02	*0.210*
BUN (mg/dL)	8.2 - 25	10.29	10.81	0.27	*0.271*
UA (mg/dL)	–	0.67	0.62	0.01	*0.311*

TP, total protein; T-Bil, total bilirubin; Alb, albumin; Cre, creatinine; BUN, blood urea nitrogen; UA, uric acid; SEM, standard error of the mean.

Nitrogen balance

Table 4 provides the indicators of nitrogen metabolism. In this study, there was a notable increase in both ND and NPU in the E diet as compared to the C diet. No variations were observed in the remaining NB parameters as an effect of the dietary treatment.

Table 4. Nitrogen balance of growing pigs

Nitrogen balance, g/head/day	C	E	SEM	*P-value*
NI	63.34	66.75	2.41	*0.915*
Fecal N content	9.34	9.44	0.07	*0.192*
Urinary N content	26.79	28.24	1.03	*0.670*
TNE	36.14	37.68	1.09	*0.037*
ND	53.99	57.31	2.35	*0.048*
NR	27.20	29.07	1.32	*0.151*
Apparent digestibility, %				
Digestibility of N	85.02	85.68	0.47	*0.238*
NE as % of intake	57.02	56.43	0.42	*0.144*
CTTAD	0.85	0.86	0.01	*0.760*
CAM	0.43	0.44	0.01	*0.870*
NPU	42.97	43.56	0.42	*0.032*

NI, nitrogen ingested; TNE, total nitrogen excretion; ND nitrogen digested; NR, nitrogen retained; NE, nitrogen excreted; CTTAD, coefficient of total tract apparent digestibility; CAM, coefficient of metabolizability; NPU, net protein utilization; SEM, standard error of the mean; $P<0.05$, significant difference between means; $P<0.01$, distinctly significant difference between means; $P\leq 0.001$, highly significant difference between means.

The TNE, which is a crucial indicator for evaluating N_2O emissions, in the current investigation, was higher (4.3%, $P<0.05$) in pigs fed with millet diet and positively correlated with ND. A potential explanation might be that various combinations of cereals exert distinct effects on feed composition and utilization, impacting not only nutritional aspects but also the intestinal environment.

It is well known that global pork consumption is increasing, and it is widely recognized that pig farming plays a crucial role in supplying a substantial amount of high-quality food (Moeller & Crespo, 2009). Nonetheless, the pig farming industry is also a significant contributor to pollution, though its impacts are relatively limited (Hörtenhuber et al., 2023).

N_2O, CO_2 exhaled prediction and e-CH_4 and CH_4 manure production of growing pigs

Elevated levels of CH_4, CO_2, and N_2O, recognized as greenhouse gases (GES) with significant implications for the phenomenon of climate change, are prompting significant environmental apprehensions (Cociș & Surdu, 2021). Achieving an accurate estimate of GES from livestock farms, especially in the case of pig farming, is a challenging issue that necessitates an integrated approach.

In this paper, we proposed to develop a model for estimating GES emissions. This model will be based on equations found in the specialized literature, incorporating parameters derived from the biological tests conducted at the IBNA Balotești, Experimental Biobase. Evidence suggests that the reduction of GES emissions from respiration, enteric fermentation, and manure, as well as their management, can be achieved through carefully designed pig nutrition strategies (Hăbeanu et al., 2019b; 2020; Hörtenhuber et al., 2023).

Microorganisms convert nitrates into N_2 and N_2O gas *via* denitrification. In practical terms, as indicated by Wang et al. (2018b) this process effectively closed the nitrogen cycle. According to Philippe & Nick (2014) cited by Habeanu et al. (2020) under farm conditions, the release of N_2O could reach up to 25% during the decomposition of pig manure.

According to Rajagopal & Béline (2011) O_2 facilitates the release of N_2O, which is 310 times more potent in inducing a warming effect in the atmosphere compared to CO_2.

The data about the exhaled N_2O, CO_2, e-CH_4, and CH_4 from pig manure are presented in Table 5. When comparing the C group to the E group, the N_2O production, expressed as g Eq. CO_2, showed a slightly decrease of 2.5% ($P>0.05$). Although the differences were not statistically significant, our observations may be attributed to the increased neutral detergent fibre (4.9%), and acid detergent fibre (3.7%), consumption.

The values of the CO_2 originating from animal respiration (maintained in a closed shelter with automatically monitored microclimate factors) were comparable between C and E groups.

As it is well-known, e-CH_4 is produced through the microbial fermentation of mainly hydrolysed food carbohydrates like cellulose, hemicellulose, pectin, and starch. According to Kebreab et al. (2006) these "fermentative" processes occur in the intestines of animals (the caecum and colon in particular) and in manure that has been stored for a long time. Table 5 shows that the emission of e-CH_4 differed significantly between the E and C groups, while CH_4 produced by manure was not considerably affected by the treatment. As presumed, a significant Pearson correlation (*r >0.75*) was found between feed intake and its components, most notably fibre and CH_4 production. There was a significant negative correlation ($P<0.05$) between ADG and both enteric and faeces CH_4 emissions.

Table 5. N_2O, CO_2 exhaled prediction and e-CH_4 and CH_4 manure production of growing pigs

N_2O and CO_2 exhaled prediction	**C**	**E**	**SEM**	***P-value***
N_2O, g Eq. CO_2	16.87	16.44	0.30	*0.128*
CO_2 emitted, g/head/day	1.63	1.64	0.01	*0.291*
e-CH_4 and CH_4 manure production				
e-CH_4, g Eq. CO_2/had/day	45.83	39.05	4.79	*0.030*
e-CH_4, g/kg ADG	42.00	32.86	6.46	*0.017*
e-CH_4, g/kg DM	19.94	16.91	2.14	*0.028*
CH_4 g Eq. CO_2/had/day	70.96	65.99	3.51	*0.144*

e-CH_4, enteric methane; ADG, average daily gain; DM, dry matter; SEM, standard error of the mean; $P<0.05$, significant difference between means; $P<0.01$, distinctly significant difference between means; $P\leq 0.001$, highly significant difference between means.

CONCLUSIONS

Millet cereal can be a complementary alternative to corn, traditionally used to feed monogastric. Based on our experimental results and due to its drought tolerance, we suggest considering the expansion of cultivated areas, particularly in arid regions and on marginal lands. Therefore, using 25% millet in the diet of growing pigs leads to significant improvement in growth parameters without negatively affecting the plasma protein profile. Due to the increase in feed consumption and specific nutrients, notably fibre, in pigs fed a millet diet, there is an observed increase in the total amount of excreted nitrogen. Moreover, there was a slight increase in N digestibility, associated with a reduction in N_2O emission, compared to the control group. Conversely, in the case of exhaled CO_2, the estimated value shows a slight increase. A notable reduction in e-CH_4 was observed, while CH_4 production in manure was not significantly affected by millet addition. There was a significant correlation between e-CH_4 and CH_4 in manure with feed intake and its components, particularly fibre. Finally, a significant negative correlation was found between ADG and CH_4 and CO_2 emissions.

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INVESTIGATIONS ON THE EFFECT OF VITAMIN SUPPLEMENTS ON BODY WEIGHT, HAEMATOLOGICAL AND BIOCHEMICAL INDICES IN QUAIL

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Abstract

The research was carried out on 40 Japanese quails (Coturnix japonica), they were divided into two groups of 20 quails each, control and experimental. Both groups were given the same forage regime, combined quail forage, growth phase. The experimental group additionally received a supplement of vitamin C (L-ascorbic acid) and vitamin E (DL-alpha tocopheryl acetate), both vitamins in a dose of 1000 mg/kg of combined feed. The duration of the experiment was 6 weeks. The investigations performed were: body weight, hemogram and some biochemical indices. These investigations were carried out at 21 days and the end of the experimental period, i.e. 42 days. At 21 days and 42 days, significant differences were observed in the individuals of the experimental group: increases in erythrocyte count, haemoglobin and haematocrit were observed. Biochemical investigations showed increases in total protein, alkaline phosphatase and calcemia. Decreases in AST, ALT, blood glucose and creatinine were observed in the experimental group. The body weight was statistically significantly higher in the group receiving the feed diet with vitamin C and E supplementation.

Key words: *biochemistry, body weight, haematology, quail, vitamin C, vitamin E.*

INTRODUCTION

Globally, the concern of growing healthier and more productive poultry is a desideratum that more and more breeders are trying to reach. This is particularly important because poultry meat, along with meat from other species, is a major source of protein worldwide (Petcu, 2013; Petcu, 2015). Thus, in the poultry industry for the best possible results in terms of productivity and reproducibility, many additives are added in animal feed (Marin et al., 2010; Marin et al., 2015; Bălăceanu, 2017; Abd El-Hack et al, 2022; Ghimpețeanu, 2022). In several specialized works the idea of using substances derived from natural sources as additives to replace the use of antibiotics is suggested in order to obtain safe food for consumers (Mitrea, 2003; Gonciarov, 2004; Petcu, 2007; Savu, 2013). The supplementation of the feed ration with different vitamin adjuvants is particularly important to support the metabolic processes of the organism (Ghiță et al., 2021).

Vitamin complexes, in particular those containing vitamin C and vitamin E, play a particularly important role as antioxidants, these two vitamins preventing lipid peroxidation. Vitamin E acts as an oxygen free radical scavenger. Vitamin C, also plays its role as an antioxidant, by reacting with peroxide radicals and at the same time works as an adjuvant for vitamin E (Surai, 2002; Kurutas, 2016; Predescu, 2018). There are numerous researches emphasizing the role and effects of vitamins C and E on the body homeostasis but also on the reproductive performance of animals (Pop et al., 2006; Răduță et al., 2017). Vitamin E is closely involved in the synthesis of several hormones involved in fertility. Deficiencies in vitamin E can materialize in malfunction of the reproductive system, but also in a poor response to the action of stressors (Mălăncuș et al., 2024). Also in the literature, numerous works are cited that emphasize the importance of vitamin C, as an adjuvant in egg production but also for hatching percentage. Several researchers have emphasized that supplementation of feed with vitamin E and vitamin C, together or separately, is a beneficial practice to eliminate the negative effects of stress, thus for better growth performance (Akinyemi et. al., 2003; Mălăncuș et al., 2022).

The aim of this study is to observe the effect of high doses of vitamin C and vitamin E on growth as well as haematological and biochemical parameters in quail.

MATERIALS AND METHODS

The experiment was carried out on the biobase of the Faculty of Veterinary Medicine in Bucharest in 2023. A number of 40-day-old Japanese quail (*Coturnix Japonica*) were used in the experiment. They were randomly divided into two groups, control and experimental, with 20 birds per group. Both control and experimental birds were kept in cages (0.70 m/0.50 m) with 10 birds in each cage. Microclimate conditions were the same for both groups. The temperature was maintained at 27ºC during the 6 experimental weeks. Water and feed were given *ad libitum*. The forage ration was purchased from the National Research and Development Institute for Animal Biology and Nutrition (IBNA) Balotești and consisted of combined feed for the quail, growth phase. The ingredients of the feed ration were: cereals, soybean meal, corn gluten, monocalcium phosphate, calcium carbonate, salt, amino acids and vitamin-mineral premix.

Nutritional values for the feed ration are: crude protein 22.50%, metabolizable energy (Kcal/kg) 3140, methionine 0.64%, methionine + cystine 0.98%, lysine 1.33%, calcium 0.96%, phosphorus 0.75%, choline 0.03%, salt 0.40%. Both groups were fed the same diet. The experimental group additionally received a supplement of vitamin C (L-ascorbic acid) and vitamin E (DL-alpha tocopheryl acetate), both vitamins at a dose of 1000 mg/kg of combined feed. Individuals were weighed twice, 3 weeks after the start of the experiment and at the end of the 6 weeks, using a Partner electronic balance. Blood samples were collected at 21 and 42 days, the experimental period, from each individual in both control and experimental groups. Blood was collected by subaxillary vein puncture from where 1 ml of blood/puncture was collected. From the blood samples the following determinations were performed: erythrocyte count, haemoglobin, haematocrit, then the derived erythrocyte constants (MCV MCH and MCHC) were calculated. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total protein (TP), calcium (Ca), glycemia (GLU) and creatinine (CREA) were performed as biochemical investigations. Hematological investigations were performed using an IDEXX ProCyte Dx apparatus, and a VetTest Chemestry Analyzer was used for biochemical investigations. All data obtained were tabulated and statistically interpreted using Student t test.

RESULTS AND DISCUSSIONS

Regarding body weight, both after 21 days and after 42 days, differences could be observed in the experimental group. Its mean body weight was 2.19% higher after 21 days and 5.26% higher after 42 days ($p<0.05$) than the values recorded in the control group (Figure 1 and Figure 2). This aspect shows a better weight gain in the group that received the feed with vitamin C and vitamin E supplementation.

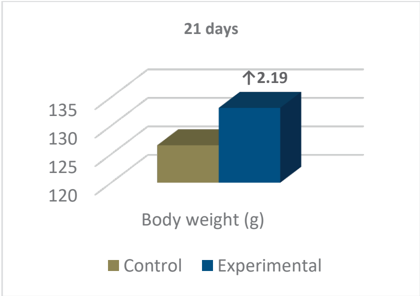

Figure 1. Mean values of body weight investigations in quails at 21 days of the experiment ($p<0.05$)

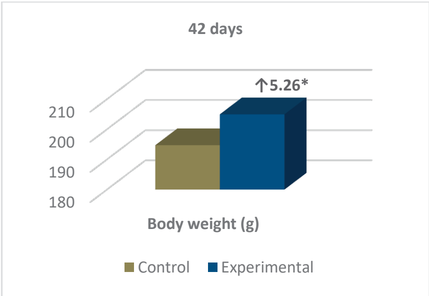

Figure 2. Mean values of body weight investigations in quails at 42 days of the experiment ($p<0.05$)

The two vitamins are particularly important as they respond to stress due to their antioxidant properties and vitamin C's ability to increase the utilisation of corticosteroids released during stress phases (Phoprasit et al., 2014; Ghiță et al., 2015). Also, the requirements of animals for these two vitamins, especially under stress, may be much higher than the requirements normally fed through feed. It is well known that stress can lead to demineralization but also to the release of some vitamins from various tissues and their excretion (Haq et al., 2016). The interaction between vitamin C and vitamin E is of importance from both a pathophysiological and animal welfare point of view, thus of great importance for food safety. Various research in the field shows that under various temperature conditions, vitamin C and vitamin E added to the feed ration in quail promotes the well-being of the animals, leading to positive growth gain (Ali et al., 2012). Consecutive to the addition of vitamin C and vitamin E to the feed ration, as observed by other researchers, the body weight in the experimental group is higher. Providing an antioxidant barrier enables the body to effectively counteract the effects of oxidative stress and thus improve metabolism. An intake of vitamin C and vitamin E is necessary to obtain healthy individuals, either for use as breeders or to ensure an adequate carcass in the case of food birds. In several studies carried out over the years, the problem of loss of constitutive juices in the muscle of poultry has been reported. In the same studies, it has been hypothesized that this can be corrected by supplementing the feed with antioxidants at increased concentrations.

The results of the haematological and biochemical investigations emphasize the beneficial effect of supplementation with vitamin C and vitamin E (Kabir et al., 2013; Lin J., 2014). Increased values of erythrocyte count, haemoglobin and haematocrit can be observed in the experimental group, both at 21 days and at the end of the experimental period.

After 21 days from the start of the experiment, some changes were observed concerning the haematological parameters: the mean erythrocyte count was 11.32% higher in the experimental group ($p<0.05$), haemoglobin and haematocrit had higher mean values than the control group, haemoglobin increasing by 6.25% and haematocrit by 3.3%.

The derived erythrocyte constants showed in the group of birds supplemented with vitamin C and vitamin E, some changes compared to the control group. The MCV was lower by 7.14% and the MCH by 4.07%. The mean erythrocyte haemoglobin concentration was higher in the experimental group, increasing by 2.71%

Similar results were also obtained at the end of the experimental period: the mean erythrocyte count was 19.65% higher in the experimental group ($p<0.05$), also haemoglobin and haematocrit had higher mean values, 7.57% and 6.45% respectively, compared to the mean values recorded in the control group ($p<0.05$).

The secondary erythrocyte constants MCV and MCH showed decreasing trends in the experimental group, 11.11% and 10.17%.

The mean erythrocyte haemoglobin concentration was higher in the control group, the mean of the determinations being 1.02% higher than in the vitamin-supplemented group (Table 1 and Table 2).

Table 1. Mean values of haematological investigations in quails at 21 days of the experiment

Parameter 21 days	**Control Group**	**Experimental Group**	**Percentage (%)**
E **x 10^6 / μl**	5.3	5.9	**↑11.32***
Hb **g/dl**	14.4	15.3	**↑6.25**
HTC **%**	30	31	**↑3.3**
MCV **μ^3**	56	52	**↓7.14**
MCH **pg Hb/E**	27	25.9	**↓4.07**
MCHC **g Hb/dl E**	48	49.3	**↑2.71**

*P<0.05 – significant differences

Table 2. Mean values of haematological investigations in quails at 42 days of the experiment

Parameter 42 days	**Control Group**	**Experimental Group**	**Percentage (%)**
E **x 10^6 / μl**	5.14	6.15	**↑19.65***
Hb **g/dl**	15.2	16.35	**↑7.57***
HTC **%**	31	33	**↑6.45***
MCV **μ^3**	60.3	53.6	**↓11.11**
MCH **pg Hb/E**	29.5	26.5	**↓10.17**
MCHC **g Hb/dl E**	49	49.5	**↓1.02**

*P<0.05 – significant differences

The increased values of erythrocyte count, haemoglobin and haematocrit are explained by the anti-oxidant effect of vitamin E and vitamin C (Figure 3 and Figure 4).

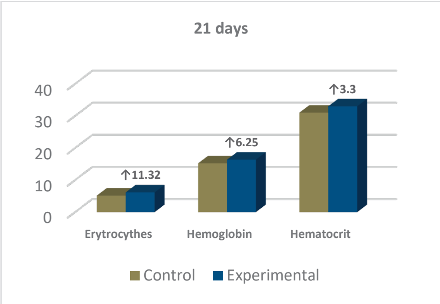

Figure 3. Eritrocytes, haemoglobin and hematocrit at 21 days of the experiment

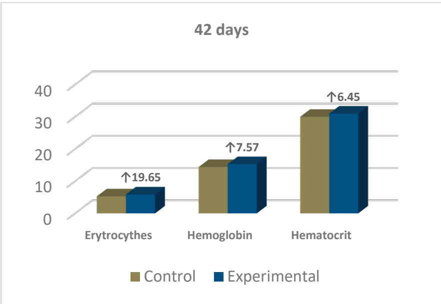

Figure 4. Eritrocytes, haemoglobin and hematocrit at 42 days of the experiment

The effect is manifested by an increase in haemato-forming activity in the haematogenous bone marrow (Răduță et al., 2017; Ghiță et al., 2022). The quantitative increase in the number of erythrocytes and haemoglobin ensures better functioning of tissues and the onset of oxygenation deficiency at the tissue level is avoided so that the skeletal muscle and the central nervous system have a correct oxygen supply. The addition of vitamin C and vitamin E to food will lead to better tissue oxygenation through increased erythrocyte counts and a prolonged lifespan. All these are explained by protecting cell membranes from the oxidative activity of various stressors (Yehmed et al., 2023). Providing better oxygenation at the tissue level also has beneficial effects for the commercial aspect of different avian products. In poultry reared for meat production, the increased oxygen supply will lead to a faster but also more harmonious body development of the carcass. The use of natural supplements instead of antibiotics in the feed ration is desirable, thus reducing the risk of antibiotic resistance, both for livestock and humans (Surai, 2002; Răduță et al., 2017).

In terms of secondary erythrocyte constants, a decrease in the MCV and in the MCH can be observed.

These decreases can be explained by a smaller volume of young erythrocytes released into the blood circulation as a result of enhanced haematopoiesis. A smaller erythrocyte volume will automatically lead to a lower mean erythrocyte haemoglobin (Figure 5 and Figure 6).

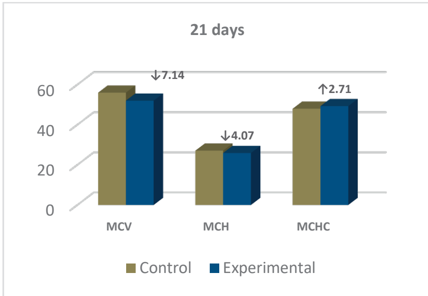

Figure 5. Mean values of haematological of indirect constants in quails at 21 days of the experiment

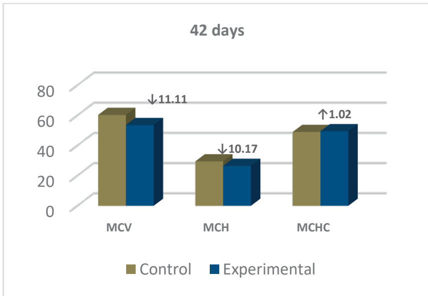

Figure 6. Mean values of haematological of indirect constants in quails at 42 days of the experiment

From the data presented in Table 3, it can be seen that 21 days after the start of the experiment, the average AST determinations in the experimental group were lower by 11.96%, and ALT showed lower values by 14.18%. Alkaline phosphatase increased by 12.24%, total protein by 11.63%, and calcium by 16.67%. Serum glucose decreased in the experimental group by 18.9% and creatinine by 5.41%. Forty-two days after the start of the experiment, the experimental group had a 12.6% lower mean AST and 35.74% lower ALT. Serum total protein increased by 22.83% (Table 4).

Table 3. Mean values of biochemical investigations in quails at 21 days of the experiment

Parameter 21 days	Control Group	Experimental Group	Percentage (%)
AST (U/L)	149,28	131,43	**↓11.96***
ALT (U/L)	32.12	28.13	**↓14,18***
ALP (U/L)	129	147	**↑12.24***
TP (g/dl)	9.03	10,8	**↑11.63***
Ca (mg/dl)	13.2	15.4	**↑16.67**
GLU (mg/dl)	212.7	172.3	**↓18.9***
CREA (mg/dl)	1.85	1.75	**↓5.41**

*P<0.05 – significant differences

Table 4. Mean values of biochemical investigations in quails at 42 days of the experiment

Parameter 42 days	Control Group	Experimental Group	Percentage (%)
AST (U/L)	151.32	132.25	**↓12.6***
ALT (U/L)	36.54	23.48	**↓35.74***
ALP (U/L)	138.3	157	**↑11.91***
TP (g/dl)	9.20	11.3	**↑22.83***
Ca (mg/dl)	14.3	16.2	**↑13.29**
GLU (mg/dl)	225.5	184.9	**↓18***
CREA (mg/dl)	1.98	1.22	**↓38.38**

*P<0.05 – significant differences

Mean serum glucose determinations in the experimental group were 18% lower than in the control group. Alkaline phosphatase (ALP) increased by 11.91%. There was also a 38.38% decrease in creatinine in the experimental group (Figures 7-10).

Similar results to the above investigations were also published by Samantha Sigolo and colleagues in 2019. In that paper, the beneficial effect of supplementing quail feed with vitamin C and vitamin E is emphasized. In birds haematological and biochemical investigations are very important indicators for their well-being. Any change in the homeostasis of the organism will be reflected in the physiological response of the birds (Scholtz et al., 2009; Saracilă et al., 2023). In line with the results obtained, our determinations have shown that feed supplementation with vitamin C and vitamin E has beneficial effects on protein, renal and liver metabolism (Cotelle et al., 2003).

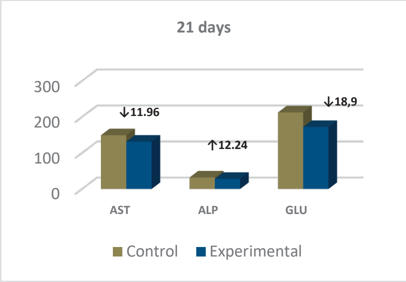

Figure 7. Mean values of AST, ALP and GLU investigations in quails at 21 days of the experiment

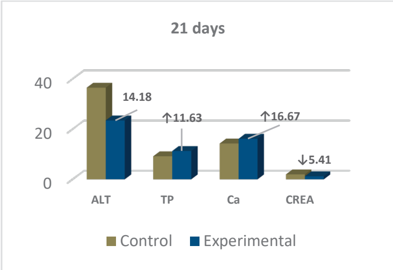

Figure 8. Mean values of ALT, TP, Ca and CREA investigations in quails at 21 days of the experiment

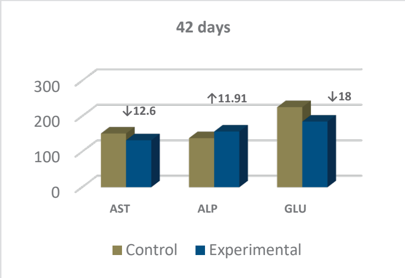

Figure 9. Mean values of AST, ALP and GLU investigations in quails at 42 days of the experiment

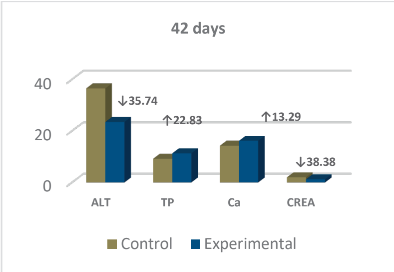

Figure 10. Mean values of ALT, TP, Ca and CREA investigations in quails at 42 days of the experiment

The low mean AST and ALT values, but increased ALP suggest an improvement in liver metabolism by reducing the permeability of cell

membranes so that the enzymes no longer leave the cytosol and are found in a lower percentage in the blood. The increase in alkaline phosphatase, most likely muscle and skeletal shows an enhancement of metabolism, a greater increase in the experimental group, an increase that may correlate with data found in body weight investigations (Goma et al., 2024).
The decrease in serum glucose concentration is explained by a decrease in its availability, as a consequence of the utilization of glucose more adequately in cellular metabolisms.
The increase of serum proteins is another aspect that suggests the improvement of metabolism, they being in higher concentration in the blood are more readily available for utilization by various systems in the body.
The property of muscle proteins to attract water and hold it at the cellular level is particularly important for meat quality (Petcu, 2015). The correlation between meat quality and muscle antioxidant resistance is well known. Differences in antioxidant defence systems in several animal species have been reported in several literature papers, with different muscle groups showing calpain damage, and protein lysis, these abnormalities being influenced by proteolysis (Cotelle et al., 2003; Haq et al., 2016).

CONCLUSIONS

As a result of supplementation with vitamin C and vitamin E in the experimental group, the body weight was 5.26% higher than in the control group. The supplementation of vitamin C and vitamin E in quail feed leads to an improvement in haematological parameters: erythrocyte count, haemoglobin, and haematocrit, the increases being statistically significant. The mean erythrocyte volume and mean erythrocyte haemoglobin show a decreasing trend, due to the smaller volume of young erythrocytes. Results of biochemical investigations showed decreases in AST, ALT, ALP and glucose in the experimental group. Mean increases in total protein and calcemia were recorded in the group fed the vitamin C and vitamin E supplements.

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THE INFLUENCE OF L-CARNITINE ON THE PRODUCTIVITY OF YOUNG RABBITS

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Abstract

In the studies, the bioadditive "CarnEon 50" was used, recommended for optimizing the intensive fattening of young monogastric animals to balance their ration with carnitine effectively. The research aimed to find out the effectiveness and safety of the use of a bio-additive as an element of intensive rabbit meat production. Research methods - zootechnical, laboratory, statistical. A compound feed recipe based on local feed ingredients was developed for intensive fattening of young rabbits of the newly created chinchilla-like type. It was established that the addition of "CarnEon 50" to the diet of fattening young rabbits in the amount of 150, 200 and 250 g/t increased their productivity at the age of 90 days, in particular, live weight - by 2.9-6.8%, average daily gains - by 4.2-10.5%, lifetime waist width (an indicator of meatiness) by - 3.04.5%, feed conversion improved by 2.5-3.9%. It is shown that the use of a bioadditive in the amount of 200-250 g/t of compound feed in the rations of young rabbits during intensive production of rabbit meat makes it possible to reduce direct costs for the production of 1 ton of rabbit meat by 20,94 USD and increase the profitability of production by 2%.

Key words: *bioadditive, fattening and slaughter indicators, intensive rabbit breeding, integrated assessment index, local fodder.*

INTRODUCTION

Modern industrial rabbit breeding is a source of valuable dietary meat, fur, leather, etc. (Petrescu & Petrescu-Mag, 2018). This is one of the most promising livestock industries, as rabbits are characterized by high fertility, early maturity, lack of seasonality in reproduction, intensive growth of young animals, and have one of the highest feed conversion rates among farm animals (Bashchenko & Luchyn, 2019; Wu, 2022). To obtain high rabbit productivity, it is important to use breeds and mixtures with high genetic productivity potential (Bojko et al., 2022) and take all necessary technological measures to realize this potential: a complete and balanced diet, controlled conditions of detention, etc. (Colin, 1993; Bashchenko et al., 2020). One of the ways to solve the problem of dietary balance is to use biologically active substances in the process of feed production, which will ensure high-quality rabbit production with the use of traditional ingredients as feed additives. They include vitamins and minerals, the absence of which in the diet leads to diseases and growth inhibition; amino acids, in particular lysine and methionine, which regulate protein metabolism (Belenguer et al., 2005); probiotics, antioxidants (Dalle Zotte & Szendro, 2011); biologically active substances of plants that have a positive effect on the gastrointestinal system, etc. (Mancini & Paci, 2021; Sedilo et al., 2022). L-carnitine performs key functions in animal metabolism, in particular, it plays an essential role in fatty acid metabolism (Flanagan et al., 2010). In intensive livestock farming technologies, L-carnitine has a multifunctional purpose, which includes: growth stimulation, improvement of the immune system, antioxidant effects, etc. (Ringseis et al., 2018b; Liu et al., 2020). Under the influence of carnitine in the diet of lactating cows, an improvement in hematological blood parameters was found (Kononov et al., 2021; Kononov et al., 2022); studies (Meyer et al., 2021; Danesh Mesgaran et al., 2021; Ghaffari, 2021) showed an improvement in the immune status and a decrease in the level of inflammation in cows during active lactation. The use of carnitine supplementation had a positive effect on nitrogen metabolism (Ringseis et al., 2018a), meat quality, and piglet growth in intensive feeding (Chen et al., 2008). The authors (Golzar et al., 2011) have comprehensively described the functional effects of carnitine under intensive chicken-rearing technologies. There are few data on the effect of carnitine on rabbit performance, in particular, the hepatoprotective effect of carnitine under conditions of parenteral administration is described (Ebeid et al., 2023), and the positive effect of carnitine in high-energy diets on feed conversion and blood parameters of rabbits is revealed (Ayyat et al., 2021).

Thus, the study of the peculiarities of application, the study of the productive effect and safety of various feed additives in rabbit fattening remains relevant. That is why the aim of the research was to substantiate and develop a scheme for the use of "CarnEon 50", to determine the optimal doses of this additive in the diets of young rabbits of chinchilla type for intensive cultivation, its effect on productivity, lifetime meatiness and slaughter yield of animals, to establish the degree of feed conversion, safety of use, profitability and economic efficiency in feeding young rabbits.

MATERIALS AND METHODS

The study of the effect of L-carnitine in the composition of the dietary supplement "CarnEon 50" on the metabolism of young rabbits of three-breed crossbreeds of Chinchilla, Flandr, and Termon white (a newly created type of Chinchilla, NCCT) was carried out at the Carpathian State Agricultural Research Station of the Institute of Agriculture of the Carpathian Region of the NAAS using intensive rabbit production technologies (Bojko et al., 2022). For this purpose, 6 groups of young rabbits aged 25 days, 10 animals in each group (5 males + 5 females) with an average weight of 494-512 g were formed by the method of pair analogues (Table 1).

The preparatory period of the experiment lasted 5 days, the main investigations were carried out for 60 days by comparative analysis of the growth intensity of young rabbits from 30 to 90 days of age based on the determination of absolute, relative and average daily growth; feed conversion, lifetime meat and slaughter indicators.

Table 1. Experiment scheme

Group	Nature of feeding young rabbits (NCCT), n = 10
1 (control)	Basic diet (BD), without dietary supplements
2 (experimental)	BD + 100 g CarnEon 50» per 1 tonne of finished feed
3 (experimental)	BD + 150 g CarnEon 50» per 1 tonne of finished feed
4 (experimental)	BD + 200 g CarnEon 50» per 1 tonne of finished feed
5 (experimental)	BD + 250 g CarnEon 50» per 1 tonne of finished feed
6 (experimental)	BD + 300 g CarnEon 50» per 1 tonne of finished feed

The feeding technology is complete granulated feed with free feeding. Local ingredients were used in the basic diet (BD): barley turf (10%), oat turf (10%), wheat bran (10%), sunflower meal (20%), alfalfa flour (35%), table salt (0.4%), and premix (3.1%). 1 kg of this fodder costing 0.29 USD contains 0.88 kg of dry matter, 175.0 g of crude protein and 160.0 g of crude fibre, providing animals with 9.19 MJ of metabolizable energy. The diets for fattening young animals during the experiment were calculated by structuring fodder according to European standards for intensively growing young rabbits (Maertes et al., 2004).

L-carnitine in the form of the biological additive "CarnEon 50" was pre-mixed thoroughly with the premix and introduced into the fodder mixture according to the experimental scheme.

The biological additive CarnEon 50 used in the study contains carnitine (48-52%) and calcium carbonate. It enriches animal, poultry, and fish feed with L-carnitine, as its endogenous synthesis covers up to 25% of the daily animal requirement. In contrast, the carnitine content in plant-based fodder is insignificant (Golzar et al., 2011).

The research was conducted by modern methodological approaches and in compliance with relevant requirements and standards, in particular, they met the requirements of DSTU ISO/EC 17025:2006 (ISO/IEC 17025:2005, IDT). The animals were kept and all manipulations with them were carried out by the provisions of the "General Ethical Principles for Animal Experiments" adopted by the First

National Congress on Bioethics and the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (European convention, 1986).

To determine the objective value of fattening and meat qualities of experimental animals, the comprehensive assessment indicator (CAI) was calculated (Luchyn, 2005) by applying indicators of average daily weight gain and lumbar width using the equation:

$$CAI=5.1(K+2H) \quad (1)$$

where CAI – comprehensive assessment indicator; 5.1 and 2 – correction factors; K – average daily increase in live weight of young animals for the growing period, g;

H – width of the lower back, cm.

The economic and technological efficiency of the use of L-carnitine in the form of the biological additive "CarnEon 50" in intensive rabbit production was determined according to generally accepted methods (Mondin et al., 2021).

All the results obtained were interpreted using the methods of variation statistics and the calculation of probability criteria using Excel 2007.

RESULTS AND DISCUSSIONS

Rabbit nutrition involves several mechanical, chemical, and microbiological processes that ensure the consistent destruction, absorption, and uptake of feed nutrients. The nutrition of young rabbits has certain age-specific characteristics. For example, the formation of the digestive tract's ability to consume plant feed is complete at three months of age. This process is stimulated by adding several biologically active substances to the rabbit diet, including carnitine.

To study the safety and efficacy of different doses of carnitine in the diet of young rabbits of three-breed crossbreeds and the effect on their productivity, analog animals were selected by weight at the age of 25 days, which during the preparatory period of 5 days were fed a diet enriched with carnitine according to the experimental scheme (Table 1). During the preparatory period, the animals adapted to the experimental diet enriched by different amounts of carnitine. There was a tendency to change the intensity of growth of young rabbits in the experimental groups (Table 2).

Table 2. Live weight of experimental animals during the preparatory period of research (m ± m, n = 10)

Group	When setting up for the preparatory period (25 days)	After the end of the preparatory period (30 days)
1 (control)	502±13.46	714±14.39
2 (experimental)	499±14.28	717±10.28
3 (experimental)	512±14.46	707±10.67
4 (experimental)	494±11.73	687±14.05
5 (experimental)	506±12.72	693±13.67
6 (experimental)	497±13.62	677±14.53

It was found that during the preparatory period, with an increase in the amount of carnitine in the diets, the average daily weight gain of animals decreased slightly, in particular, the weight gain of rabbits of the third, fourth, fifth, and sixth experimental groups was 27.5; 27.6, 26.7 and 25.7 g, respectively, which was 9.2, 8.9, 11.8 and 15.2% less than the rabbits of the control group, whose weight gain was 30.3 g, thus the adaptation of young rabbits to the experimental conditions was optimal.

To determine the effectiveness of the use of the bioadditive "CarnEon 50" on the productive characteristics of young rabbits, the average live weight of animals at 90 days of age was evaluated using different doses of carnitine for 60 days. It was shown that optimization of the diet of young rabbits by adding different doses of carnitine had a positive effect on the average live weight of animals in all experimental groups (Table 3).

Table 3. Growth rate of young rabbits (M ± m, n = 10)

Group	Live weight of 1 animal		Average daily rates increments, g
	at 30 days of age, g	at 90 days of age, g	
1 (control)	714±14.39	2692±39	36.0±0.8
2 (experimental)	717±10.28	2715±47	36.3±0.9
3 (experimental)	707±10.67	2770±39	37.5±0.8
4 (experimental)	687±14.05	2875±37*	39.8±0.7*
5 (experimental)	693±13.67	2840±52*	39.0±1.0*
6 (experimental)	677±14.53	2705±53	36.9±1.1

Note: *$p<0.05$.

The maximum live weight of one individual at 90 days of age was in groups 4 and 5 with the use of 200 and 250 g/t of carnitine supplementation, respectively, and was 2875 and 2840 g compared to 2692 g in the control, i.e. an increase in the live weight of rabbits of both groups by 7% was recorde.

An important objective characteristic for assessing the effectiveness of dietary supplements, especially in conditions of intensive rabbit production, is the value of average daily live weight gain because this indicator makes it possible to establish the nature of the impact of changes in the diet on rabbit productivity. The highest average daily weight gain for 30-90 days was observed in animals of the fourth and fifth groups, with the use of 200 and 250 g/t of carnitine supplementation - 39.8 and 39.0 g, which is 11 and 10% higher than in the control group, respectively, slightly lower were these indicators in rabbits of the third and sixth experimental groups, with the use of 150 and 300 g/t of carnitine supplementation - 37.5 and 36.9 g, which exceeds the corresponding increases in the control group by 2.5 and 4.1%, respectively. At the same time, the average daily weight gain of animals in the second group was within the control values, and the average daily weight gain in the fourth and fifth groups of young rabbits had a significant difference from the control ($p<0.05$).

An important characteristic of the feeding productivity of young rabbits under intensive fattening technologies is the width of the lower back - an important vital index that allows to assess the potential of meatiness of animals and, if necessary, to adjust the diet to ensure maximum animal productivity determined by genotype. According to the data in Table 4, the width of the lower back in rabbits at 3 months of age increased slightly with the optimization of the diet with different doses of carnitine and was highest in animals of the fourth group receiving 200 g/t of carnitine supplementation, where it was 6.9 cm, which is 5% more than in the control.

When the diet of experimental rabbits was optimized with lower and higher doses of carnitine in the composition of the additive "CarnEon 50" in the amount of 100, 150, 250, and 300 g per tonne of finished feed, the lifetime lumbar width of experimental animals did not differ significantly from the control (Table 4).

Table 4. Fattening and slaughter indicators of young rabbits at 90-day age, ($m \pm m$, $n = 10$)

Group	Width of lower back, cm	Weight of a paired carcass, g	Slaughter output, %	Feed costs per 1 kg of growth, kg	CAI
1 (control)	6.6±0.1	1336±17	49.5	3.85	251
2 (experimental)	6.7±0.11	1362±25	50.2	3.85	253
3 (experimental)	6.8±0.07	1406±19	50.7	3.75	261
4 (experimental)	6.9±0.07	1460±18**	50.8	3.7	273
5 (experimental)	6.8±0.09	1433±24**	50.5	3.7	268
6 (experimental)	6.6±0.08	1362±24	50.4	3.8	255

Note: **$p<0.01$.

The analysis of slaughter indicators, such as the weight of a paired carcass, showed a somewhat uneven increase in the animals of the experimental groups. Thus, the young rabbits of the third, fourth, and fifth groups, which received 150, 200, and 250 g/t of carnitine supplementation, outperformed the animals of the control group by 70, 124, and 94 g, respectively. Animals of the second and sixth groups, whose diets were enriched with 100 and 300 g/t of carnitine supplementation, slightly outperformed the control group in terms of paired carcass weight.

The slaughter output of young rabbits, an important qualitative characteristic of intensive rabbit breeding, positively changed in all experimental groups. Analysis of the slaughter output of young rabbits showed an advantage of animals of the third, fourth and fifth groups over animals of the control group by 1.0-1.3%.

Fodder costs for intensive technologies of fattening young rabbits are determined by the characteristics of animal metabolism and are a factor in the efficiency of feed conversion. The analysis of the cost of finished fodder per unit of gain showed effective feed assimilation by animals of the fourth and fifth groups, whose diet was enriched with 200 and 250 g/t of carnitine supplementation, as feed consumption was only 3.70 kg, which is 4% lower than in the control. Animals in the third group, which received 150 g/t of carnitine supplementation, consumed 3.75 kg of feed compared to 3.85 kg of animals in the control group. Feed consumption in animals of the second and sixth groups, whose diets were enriched with 100 and 300 g/t of carnitine supplementation, was 3.85 kg and 3.80 kg, which was at the level of the control.

Improving the metabolism of rabbits due to the use of different doses of carnitine supplementation in the diet of intensive fattening of young animals naturally led to an increase in the comprehensive assessment indicator (CAI), which is calculated based on the average daily weight gain and lumbar width. The highest values of this indicator were obtained for young animals of the fourth and fifth groups, which received 200 and 250 g/t of carnitine supplementation, respectively, and amounted to 273 and 268, while for animals of the control group, this indicator was 251.

Thus, the use of carnitine supplementation in the diet of fattening young rabbits at doses of 150, 200 and 250 g per tonne of finished feed increased live weight by 2.9-6.8% at 90 days of age, average daily gain by 4.2-10.5%, lifetime loin width (meatiness) by 3.0-4.5%, and feed conversion improved by 2.5-3.9% compared to the control.

Table 5. Economic efficiency of using different amounts of bioadditive "CarnEon 50" in feeding young rabbits

Economic indicators	Group					
	1	2	3	4	5	6
The cost of 1 kg of dietary supplement, USD	-	39.27	39.27	39.27	39.27	39.27
The cost of 1 tonne of mixed feed, USD.	287.99	291.91	293.88	295.84	297.80	299.77
Feed costs per 1 kg of weight gain, kg	3.85	3.85	3.75	3.7	3.7	3.8
The cost of feed per 1 kg of live weight gain, USD	1.11	11.12	1.10	1.09	1.10	1.14
Cost of 1 kg of rabbit meat, USD	1.58	1.61	1.57	1.56	1.56	1.63
Sales price of 1 kg of live weight of rabbit meat, USD	2.09	2.09	2.09	2.09	2.09	2.09
Net income 1 kg of rabbit meat, USD.	0.51	0.49	0.52	0.53	0.53	0.46
Profitability, %	32	30	33	34	34	29

The best indicators of fattening productivity at 90 days of age were obtained with the addition of 200 g of "CarnEon 50" per tonne of finished fodder, probably due to the effectiveness of the physiological effect of carnitine on the digestion and metabolism of monogastric herbivores, which was manifested in better feed intake. The live weight of rabbits in this experimental group increased by 183 g, average daily weight gain by 3.8 g, loin width by 0.3 cm, and feed conversion improved by 150 g.

Optimization of the diet of young rabbits under intensive fattening conditions by adding different amounts of L-carnitine as part of a dietary additive led to positive changes in animal prodctivity but also caused a rise in fodder prices.

That is why an economic analysis of the effectiveness of the use of bio-additive with L-carnitine as a component of the intensive technology of rabbit meat production was conducted. The data of the economic analysis, presented in Table 5, show that feed costs per 1 kg of growth slightly decreased in all experimental groups (by 4%) compared to the control, except for 2 experimental groups of animals, where the level of feed costs remained unchanged compared to the control values. At the same time, a natural increase in the cost of 1 ton of ready-made feed was found in all experimental groups (from 287.99 USD to 299.77 USD), caused by the optimization of feed nutrition due to the addition of different doses of bio-additive.

The use of "CarnEon 50" bioadditive increased the fattening productivity of rabbits with a simultaneous decrease in the cost of feed per 1 kg of growth by 2-5%, the exception was the sixth group of rabbits that received a diet enriched with 300 g/t of the additive, for which an increase in the cost of 1 kg of feed was noted by 1.5%. Similar data were obtained for the cost of feeding young rabbits, which decreased in all experimental groups compared to control animals (from 1.58 to 1.56 USD), the exception was the cost of feeding animals of the sixth group, which amounted to 1.63 USD.

The increase in the cost of feed and the cost of feeding rabbits naturally affected the indicators of net profit and profitability of production. The highest profitability of production based on direct costs was recorded for animals of the fourth and fifth groups and amounted to 34%. In addition, the animals of these groups had better performance indicators, which allows us to recommend the doses of "CarnEon 50" bioadditive with carnitine of 200 and 250 g/t as an important element of intensive production of rabbit breeding products.

Thus, optimization of the rations of young rabbits under the conditions of intensive production of rabbit meat by adding to the basic compound feed "CarnEon 50" bioadditive in doses of 200 and 250 g/t makes it possible to make the production of rabbit meat more efficient. At the same time, direct costs for the production of 1 ton of rabbit meat are reduced by 20.94 USD, and the profitability of production increases by 2%.

A characteristic feature of the metabolism of young rabbits of the chinchilla type is a high daily increase in live weight, which occurs due to the effective conversion of feed, but may be accompanied by an imbalance in the conversion of feed and disruption of the work of the gastrointestinal tract, since under certain circumstances this can lead to the development of enteritis, and, respectively, to a decrease in productivity (Petrescu & Petrescu-Mag, 2018; Bashchenko et al., 2020).

Adjustment of metabolism in such a case is a prerequisite for intensive production of rabbit meat and is carried out, first of all, by optimizing rations, as well as improving housing conditions, etc. (Belengueret, 2005; Wu, 2022).

The main source of energy for rabbits is the carbohydrates and fats of the feed, while young animals use the energy of the feed nutrients more intensively. In particular, during the day in the body of a 60-75-day-old rabbit, about 1/3 is deposited in protein, 2/3 in fat - 2/3 of the energy of nutrients, and in a 90-105-day-old rabbit - 1/4 and 3/4, respectively (Bashchenko & Luchyn, 2019).

Carnitine plays an important role in energy metabolism by supporting the transport of activated fatty acids to the subcellular site of β-oxidation. In particular, the administration of exogenous carnitine can limit the use of fatty acids as substrates for mitochondrial oxidation, especially when there is an increased energy demand (Flanagan, 2010). L-carnitine supplements are effective in critical periods of animal development, such as intrauterine development, lactation, and intensive growth of young animals. The concentration of L-carnitine in animals varies considerably between species, depending on the type of tissue and nutritional level (Ebeid et al., 2023).

In particular, in poultry farming, L-carnitine has a multifunctional purpose, which includes: growth stimulation, strengthening of the immune system, antioxidant effect, and improvement of poultry productive qualities (Golzar et al., 2011). The effect of carnitine on erythro- and thrombopoiesis has been shown, and an increased oxygen demand has been noted, which is associated with the effect of stabilizing cell membranes (Liu et al., 2020).

The addition of carnitine to the ration of lactating cows caused changes in the mRNA transcription of some genes associated with mitochondrial biogenesis and low-density lipoprotein synthesis, thereby accelerating liver regeneration processes and contributing to the optimization of fatty acid metabolism in animals (Ghaffari, 2021).

In the literature, there is a lot of data on the effect of exogenous carnitine, both in the diet and by parenteral administration, on improving the productivity of farm animals, but the results are very controversial (Ayyat et al., 2021; Chen et al., 2008; Ringseis et al., 2018a). That is why, when using carnitine as a feed additive, it is necessary to take into account the carnitine status of animals, which directly depends on the homeostasis of this acid in the body and includes endogenous synthesis, intake and absorption of carnitine from the gastrointestinal tract and ways of its excretion from the body (Ringseis et al., 2018b).

Many studies show that carnitine is bioavailable to animals, both ruminants and monogastrics, even as a dietary supplement, but its effect on milk or meat production is not always observed, and correlates with endogenous carnitine levels and the stage of ontogeny of the animal. However, supplementation with carnitine is a useful strategy to protect against excessive ammonia caused by the consumption of unbalanced feeds high in non-protein nitrogen or feeds high in soluble nitrogen (Kononov et al., 2022).

The positive effect of carnitine supplementation is associated, in particular, with a decrease in the level of unesterified fatty acids in blood plasma, an increase in the level of eosinophils, which indicates an increase in the regenerative properties of tissues. The potential impact of L-carnitine supplementation on immune cell function, blood cortisol concentrations, and proliferation has also been established (Kononov et al., 2021).

The inclusion of L-carnitine in the ration of highly productive Holstein dairy cows as part of the rumen enzyme-resistant preparation "Carneon 20 Rumin-Pro" contributed to an increase in their milk production and milk quality compared to the control group. The administration of L-carnitine to these cows reduced the concentration of high and low density lipoproteins in the peripheral blood. To some extent, this can be explained by the ability of L-carnitine to modulate energy metabolism and liver function (Danesh Mesgaran et al., 2021).

A several authors have studied the effect of adding L-carnitine to normal and high-energy diets of young rabbits reared under severe heat stress conditions on their productivity, physiological parameters and carcass characteristics. A significant improvement in the physiological and biochemical parameters of animals, an increase in their growth parameters and the degree of feed conversion was established. These animals showed significantly higher levels of haemoglobin, leukocytes, total protein, and glucose compared to the control, which indicates the safety and effectiveness of carnitine use when rearing them under stressful conditions (Ayyat et al., 2021; Flanagan, 2010).

CONCLUSIONS

The use of an additive with carnitine "CarnEon 50" in the main diet of intensive fattening of chinchilla-like young rabbits in doses of 150, 200, and 250 g per tonne of finished feed led to an increase in the live weight of 90-day-old animals by 2.9-6.8%, average daily gain by 4.2-10.5%, and lifetime loin width (meatiness) by 3.0-4.5%. Feed conversion improved by 2.5-3.9% compared to the control.

Animals whose diets were optimized with carnitine supplementation at doses of 200 and 250 g/t outperformed the control animals by 124 and 94 g ($p<0.01$), and the slaughter yield of young rabbits of these groups was 1.0-1.3% higher than the control.

Using 200 and 250 g/t of carnitine additive in rabbit fattening technology reduced feed costs to 3.70 kg of finished feed per 1 kg of weight gain compared to 3.85 kg in the control.

The use of the additive "CarnEon 50" in the amount of 200-250 g/t of feed in the diets of young rabbits for intensive rabbit production allows to reduce direct costs for the production of 1 ton of rabbit meat by 20,94 USD and increase the profitability of production by 2 %.

Thus, the optimization of the diet of young rabbits of chinchilla type for intensive rabbit production by adding 200 and 250 g/t of additive to the feed contributed to the improvement of lifetime meatiness and slaughter yield with a simultaneous reduction in feed costs and an increase in its conversion, which reduced the cost of rabbit production. The use of carnitine as a component of intensive fattening contributed to an increase in the resistance of young rabbits to gastrointestinal diseases and ensured the absence of animal mortality during the study.

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ASSESSMENT OF THE ANTIOXIDANT AND ANTIBACTERIAL POTENTIAL OF SOME PROPOLIS-BASED NATURAL PRODUCTS

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Abstract

Propolis has been used for centuries as a medical remedy in both humans and animals. Propolis can be found as a single basic product or as an additional compound in standardized drug formulations. The present research aimed to assess the antioxidant and antibacterial activity of four propolis-based natural products. The investigations were carried out on the following products: a propolis tincture; an aqueous propolis extract; an aqueous propolis extract with colloidal silver; and an ointment consisting of propolis, olive oil, and propolis wax. According to the obtained results, of the four products, the ointment showed the highest antioxidant activity. A higher antibacterial potential has also been demonstrated by the ointment compared to the other investigated formulas.

***Key words**: antibacterial, antioxidant, propolis-based products.*

INTRODUCTION

Propolis is commonly referred to as "bee glue", a colloquial term for the resinous material that bees gather from various plant kinds. The Greek word "propolis" has a particular sense, "pro" meaning "defense" and "polis" signifying "community" (Castaldo & Capasso, 2002).
The bioactive components of propolis provide both antioxidant and immune-protective qualities. Propolis is made up of several different compounds, which vary depending on the geographical area. Therefore, propolis contains a variety of chemicals, such as phenolic acids, alcohols, aromatic aldehydes, lignans, flavonoids, esters, diterpenes, sesquiterpenes, amino and fatty acids, as well as minerals and vitamins (Batista et al., 2012).
The main flavonoid components of propolis, galangin, pinocembrin, and pinostrobin, are linked to its antibacterial action. They interfere with the DNA genetic encoding of microorganisms and enhance the membrane's permeability of bacteria (Cornara et al., 2017). Moreover, flavonoids can function as powerful anti-bacterial compounds by blocking the secretion of nucleic acids, the adhesion and production of biofilms, and the pathogens' metabolism (Freires et al., 2016).
There are two aspects to take into account regarding the propolis's antibacterial activity. Firstly, it is associated with the immediate impact on the microbe itself, and secondly, with the immune system boost that triggers the organism's inherent defense mechanisms (Sforcin & Bankova, 2011).
The present paperwork aimed to study four natural Romanian propolis-based products, by assessing their antioxidant and antibacterial potential.

MATERIALS AND METHODS

The biological material used in this study was represented by four Romanian propolis-based products and their form of presentation and composition are shown in Table 1. To prevent any alteration or change of the product's composition, the samples were stored in their original, hermetically sealed glass containers for tinctures and aqueous extracts, and plastic containers for ointments, respectively. Additionally, all products were kept out of direct sunlight in dry space until further analyses were carried out.

Table 1. The investigated apitherapeutic products

No. crt.	Product	Form of presentation	Composition
P1	Aqueous propolis extract with colloidal silver	Aqueous extract	Aqueous propolis extract, colloidal silver 70 ppm
P2	Propolis tincture	Tincture	Ethyl alcohol, propolis
P3	Aqueous propolis extract	Aqueous extract	Aqueous propolis extract
P4	Propolis and olive oil-based ointment	Ointment	Propolis, olive oil, propolis wax

The DPPH (2, 2-Diphenyl-1-picrylhydrazyl) radical scavenging activity of the products was assessed spectrophotometrically according to Bankova et al. (2016), with certain adjustments made within the Laboratory for Quality Control of Bee Products and Diagnosis in Bee Diseases (APHIS-DIA) USAMV Cluj-Napoca because of the specifics of the studied products. This test is a widely used technique for evaluating the antioxidant capacity of different products. To conduct this analysis, the samples were prepared as follows: for the liquid products, 1/10 dilutions were made, using water as a solvent for P1 and P3, and 80% ethanol for P2, respectively. In regards to the ointment (P4) containing propolis, olive oil, and propolis wax, solubilization was required first using the following solvents: pure hexane; a mixture of hexane and methanol 70% 1:1; a mixture of hexane and ethanol 1:1; and dimethylsulfoxide (DMSO). The purpose of using several substances was to compare the degree of solubilization of the product, considering its oily-viscous consistency. Based on the results, the mixture containing methanol and hexane has been used in the study's subsequent stages.

The DPPH solution was prepared extemporaneously; thus, 1 mg of solid DPPH were dissolved in 50 ml of pure methanol, obtaining a solution with a concentration of 0.02 mg/ml; then, 0.5 ml of each diluted sample and 2.5 ml of DPPH solution were added. The blank sample consisted of 0.5 ml of methanol and 2.5 ml of DPPH solution. The samples' absorbance was read at the wavelength of λ = 517 nm. The antioxidant activity of the samples was expressed as a percentage of inhibition (% inhibition) and IC50 (the sample concentration required to inhibit 50% of free DPPH radicals).

The obtained data regarding both % inhibition and IC50 for all the investigated products (P1, P2, P3, and P4) was tested for normal distribution using the Shapiro-Wilk test, followed by ANOVA one-way and Tukey's multiple comparisons test (alpha was set 0.05).

The broth microdilution method, which has been standardized by the Clinical and Laboratory Standards Institute (2012) and well documented in the literature (Patton et al., 2006; Drago et al., 2007; Cremers et al., 2020), was utilized to evaluate the antibacterial activity. The lowest dose of an antimicrobial drug at which no microbe growth was detected in agar or broth dilution techniques is known as the minimum inhibitory concentration, or MIC (Clinical and Laboratory Standards Institute, 2012). However, the specificity of the products under study required certain modifications.

The medium used to cultivate the bacterial isolates and assess their susceptibility to the investigated products was Mueller Hinton broth (MHB).

The tested bacterial strains were clinically isolated from various skin wounds of canine, feline, and equine patients within the Microbiology Department, Faculty of Veterinary Medicine, USAMV Cluj-Napoca. The bacterial strains along with their source of origin are listed in Table 2.

Table 2. The provenance of the isolated *Staphylococcus* spp. strains

Bacterial strain	Species	Wound type
307	Canine	Avulsion of the left forelimb
199A	Canine	Skin wound of the left flank region
403C	Feline	Skin wound around the tail base
375	Equine	Postoperative abdominal skin wound (umbilical hernia)
272	Equine	Skin wound on the left stifle
273	Equine	Skin wound of the left fetlock

The bacterial suspensions of the isolated strains were realized in MHB in a 0.5 McFarland turbidity.

Four 96-well plates were inoculated as shown in Figure 1 for the antibacterial activity testing.

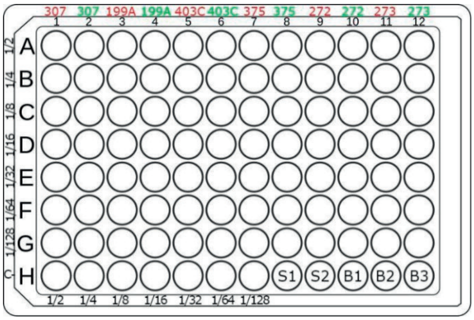

Figure 1. Plate inoculation model for determining the minimum inhibitory concentration; the red numbers represent the test columns and the green numbers the positive control columns; H row 1-7 (C-) negative control consisting in dilution of the product with sterile broth instead of bacterial suspension row; S1, S2 – saline sterility control; B1, B2, B3 – broth sterility control

The two-fold serial dilutions method was used to dilute the products (vertically from A to G); for liquid products (P1, P2, P3), the solvent was sterile saline. In the case of the ointment (P4), the product was first dissolved in 96° alcohol (1/2), after which the two-fold serial dilutions were done in sterile saline. Regarding the H row, wells 1 to 7 were named negative control wells (C-) in which 100 µL of two-fold serial dilution of the product and 100 µL of sterile broth were added. Wells H 8-12 served as sterility controls, wells S1 and S2 for the saline used for products' dilutions, and wells B1, B2, and B3 for the broth. In the odd columns (1, 3, 5, 7, 9, 11, rows A to G) representing the test columns, over 100 µL of the serial dilution of the product, 100 µL of bacterial suspension was added. In the even columns (2, 4, 6, 8, 10, 12, rows A to G), referred to as positive control columns, over the 100 µL of sterile saline used instead of diluted product, 100 µL of bacterial suspension was added. In the case of P4, two-fold serial dilution of the 96° alcohol in saline was used instead of saline in the positive control columns. After adding the bacterial suspensions, the plates were incubated at 37°C for 24 hours.

RESULTS AND DISCUSSIONS

The DPPH method was used to assess the antioxidant capacity of the investigated products. When DPPH radical reacts with various hydrogen donors, like antioxidants, it decolorizes from its dark purple hue to pale yellow (Baliyan et al., 2022). The color change was monitored using UV spectrophotometry. Determinations were made according to the following formulas:

$$\% \text{ Inhibition} = [(\text{Absorbance blank} - \text{Absorbance sample})/\text{Absorbance blank}] \times 100;$$

$$\text{IC50 sample} = [(50 \times \text{sample concentration } (\%)]/ \% \text{ Inhibition}$$

The final value for each propolis-based product is the mean of three independent determinations ± Standard deviation, as seen in Figure 2 and Figure 3.

The ointment (P4) consisting of propolis, olive oil, and propolis wax, exhibited the highest radical scavenging, with a mean % inhibition value of 94.15% ± 0.16; however, the propolis tincture's (P2) mean value of % inhibition was quite similar to P4, namely 89.24 % ± 0.29. The lowest antioxidant activity was observed among the aqueous extracts, as follows: the aqueous propolis extract (P3) recorded a mean % inhibition value of 51.27±0.84%, whereas the aqueous propolis extract with colloidal silver (P1), registered a mean value of 38.87±5.16%.

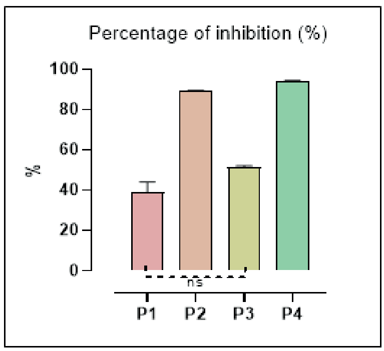

Figure 2. Graphical representation of the % inhibition of the samples

The IC50 value expresses the sample concentration required to inhibit 50% of the free DPPH radicals present in the solution. The lower this value, the higher the antioxidant capacity of the sample. As expected, the lowest mean value of IC50 was found in P4 (0.53 ± 0.0009), followed by P2 (0.56 ± 0.0019), P3 (0.98 ± 0.0158), and P1 (1.30 ± 0.1612), respectively. Overall, statistically significant differences were observed when comparing the antioxidant activity (% inhibition and IC50) of the tested samples. The results of the ANOVA one-way test indicated that there is a statistically significant difference between the compared values (Percentage of Inhibition: $F = 60.18$; $p = 0.0157$ and IC50: $F = 325.7$; $p = 0.0028$). Further analysis using Tukey's multiple comparison test revealed that there were no significant differences between the antioxidant potential of products P1 and P3 regarding both the % Inhibition ($p = 0.1477$) and the IC50 values ($p = 0.1895$). This suggests that, according to Tukey's test, the antioxidant effects of products P1 and P3 were similar and not statistically different from each other.

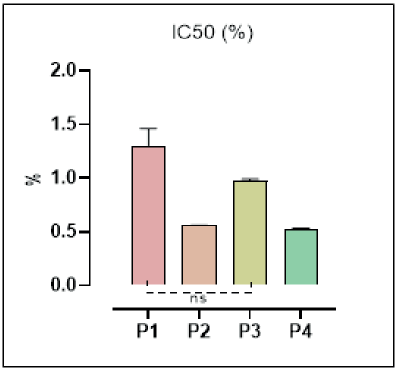

Figure 3. Graphical representation of the IC50 of the samples

The antibacterial activity of the investigated products was initially evaluated by optical density (OD) read using a multichannel spectrophotometer.

In the case of P1 and P4, the minimum inhibitory concentrations were obtained for each bacterial strain, by calculating the difference between the OD of the serially diluted product inoculated with the bacterial strain and the absorbance of the negative control (template) of the same concentration. The obtained result was compared with the corresponding positive control. The last dilution where the difference value was less than half of the positive control value was considered to be the minimum inhibitory concentration of these products.

Regarding P2 and P4, the MIC could not be determined using the calculation method described above. Both these products have a more intense color than the aqueous extracts, and if bacterial growth occurs in the wells, due to the higher turbidity, the sample will lose its initial absorbance.

Due to the difficulties in the interpretation of the obtained OD, the antibacterial activity of the investigated products was evaluated by a naked-eye assessment of the medium's turbidity. The results for P1 and P3 were comparable using both assessments. The minimum inhibitory concentrations (% of propolis-based products in saline) are presented in Table 3.

Table 3. The minimum inhibitory concentrations (%)

Product	*Staphylococcus spp.* bacterial strains (MIC; %)					
	307	199A	403C	375	272	273
P1 (Aqueous propolis extract with colloidal silver)	6.25	3.12	3.12	6.25	12.5	6.25
P2 (Propolis tincture)	3.12	12.5	3.12	6.25	3.12	6.25
P3 (Aqueous propolis extract)	6.25	25	25	25	25	12.5
P4 (Propolis and olive oil-based ointment)	1.56	6.25	6.25	3.12	1.56	1.56

Product P4 emphasized the best inhibitory activity against the tested bacterial strains, the 1.56% concentration having an inhibitory effect on the growth of 3 bacterial strains (307, 272, 273), and that of 3.12% on a single strain (375); the 199A and 403C strains proved to be the most resistant, but even so, they were inhibited by a concentration of 6.25% of the product; additionally, 5 bacterial strains were inhibited at concentrations in the range of 3.12-6.25% in the case of P1 (aqueous extract with silver), while P3 (aqueous propolis extract) inhibited one bacterial strain (307) at the concentration of 6.25% (307) and another strain (273) at the concentration of 12.5%.

As a study limitation, in the plate no. 4 designed for P4, the solvent used to dilute the product might have interfered with the bacterial growth. However, considering the increased antioxidant activity of the ointment, it is unlikely that the obtained results regarding the antibacterial potential are due to the presence of

the solvent and not to the bioactive compounds of the product.
All investigated formulas contained propolis, and the latter is described in the literature as a therapeutic agent due to its curative properties. Available data indicate that propolis exhibits a wide range of significant biological actions as a result of the presence of biologically active components, including antibacterial, antifungal, anti-inflammatory, antiviral, antitumor, hepatoprotective, cardioprotective, and immunomodulatory properties (Farooqui and Farooqui, 2012; Jansen-Alves et al., 2019; Rivero-Cruz et al., 2020; Yuan et al., 2019; Ozdal et al., 2019; Asem et al., 2020; Bhadauria et al., 2010). In addition, flavonoids and phenolic acids, recognized as protective agents against reactive oxygen species are responsible for propolis's antioxidant action. However, the average amounts of polyphenolic components in Romanian propolis extracts have been found to present considerable variation depending on the geographical area (Gatea et al., 2015; Mărghitaş et al., 2014; Dezmirean et al., 2017), the procedures used in beekeeping (Stan et al., 2011), and harvesting period (Mărghitaş et al., 2013). The extraction technique also influences the amount of polyphenols found in propolis (Oroian et al., 2020a; Oroian et al., 2020b).
The studied products displayed notable radical scavenging activity, with P4 providing the best antioxidant potential. This aspect may be due to its unique composition; olive oil is known for its phenolic content, which exerts strong antioxidant effects (Tuck & Hayball, 2002). In addition, most bioactive chemicals are poorly soluble in water, and phenolic substances are ten times less abundant in aqueous propolis extracts than they are in ethanolic propolis extracts (Mello et al., 2010; Moura et al., 2009). These findings were in accordance with the results of our study; the aqueous propolis extracts showed lower antioxidant activity than the propolis tincture (Figure 2; Figure 3).
The ability of propolis to prevent microbial growth, such as yeasts, molds, and both Gram-positive and Gram-negative bacteria, is well acknowledged (Bankova et al., 2014; Benhanifia et al., 2014; Nedji & Loucif-Ayad, 2014; Özcan et al., 2004; Anjum et al., 2019). In general, propolis has greater effects against Gram-positive than Gram-negative bacteria. The secretion of enzymes that degrade the constituents of propolis, as well as the particular structure of the outermost membrane of Gram-negative bacteria, contribute to this aspect (Sforcin, 2016; Kędzi & Hołderna-Kędzia, 2013).
The testing of the products' antibacterial activity highlighted again the strongest potential of the P4 product, namely the ointment based on propolis, olive oil, and propolis wax. This characteristic is also associated with the presence of polyphenols in olive oil, which possess antibacterial effects as well (Capasso et al., 1995). Regarding the antibacterial activity of the aqueous propolis extracts, P1, which has colloidal silver in addition to P3, recorded better values. The antibacterial activity of colloidal silver against both Gram-positive and Gram-negative bacteria was postulated by Vila-Domínguez et al. (2020). According to Barras et al. (2018), this may be explained by the damaging effect of silver on microorganisms by adhering to the chemical structures found on their surface.

CONCLUSIONS

Based on the results obtained when assessing the antioxidant and antibacterial potential of the investigated products, we consider that the use of apitherapeutic formulas in medical practice may offer a viable substitute for diminishing the incidence of antibiotic resistance. We also appreciate that the form of presentation of propolis-based products has a direct influence on the biologically active compounds' content and implicitly on the bioactive properties of the products. In addition, during the study we encountered difficulties regarding the dilution of propolis-based products with a viscous-oily consistency; therefore, conducting more research on finding the most suitable solvents for the solubilization of this type of products is crucial for obtaining conclusive results.

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THE INFLUENCE OF NATURAL FEED ADDITIVES ON THE PRODUCTIVE PERFORMANCE OF BROILER CHICKEN - A REVIEW

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Abstract

Natural feed additives for broilers have gained increasing attention in recent years due to the growing demand for sustainable poultry production. These additives are derived from plant, animal, or microbial sources and are used to enhance broiler health, improve growth performance, and optimize feed efficiency. As consumer preferences shift towards more natural and organic products, the aim of the paper was to highlight that the use of natural feed additives provides an alternative to synthetic additives and antibiotics, which are increasingly restricted due to concerns about antibiotic resistance and environmental impact. From phytobiotics and probiotics to enzymes, natural feed additives provide a wide range of benefits, including improved nutrient digestibility, enhanced immune function, and better meat quality. While challenges such as cost and variability remain, advances in research and formulation will continue to optimize their use in broiler production.

Key words*: broiler, enzymes, organic acids, phytobiotics, probiotics and prebiotics.*

INTRODUCTION

In order to improve the quality of meat, increase the economic efficiency of production and ensure its sustainability, a series of measures can be applied in broiler farms aimed at bird nutrition, genetic selection, reducing the impact of growing chickens on environmental pollution, breeding technologies that ensure the welfare of the birds.

Researchers are exploring genetic selection and gene editing techniques to develop chicken lines more resistant to common diseases such as coccidiosis and hepatic necrosis (Nagpal et al., 2012). New methods of genetic selection are also being studied to increase the efficiency of conversion of feed to meat, thereby reducing production costs and environmental impact (Rehman et al., 2020).

To ensure the sustainability of poultry production, research is focused on reducing ammonia emissions from chicken farms through changes in nutrition and manure management, in order to reduce the impact on the environment (Alagawany et al., 2016). At the same time, alternative sources of protein, such as insects or agro-industrial residues, are being investigated to create more sustainable and economical feed (Belhadj Slimen et al., 2023).

Technologies are being developed on farms to monitor the health and behavior of chickens in real time, using sensors and artificial intelligence to optimize rearing conditions (Taleb et al., 2024).

The purpose of this work is to investigate the possibilities of improving the nutrition of broiler chickens through different methods, such as the use of natural feed additives and different sources of fats and oils, in order to improve the bioproductive performances, the health and welfare of the birds and to offer safer and healthier for consumers.

MATERIALS AND METHODS

In order to provide a complete and balanced picture of the proposed subject, a wide variety of bibliographic sources were analyzed which included scientific articles, books, papers presented at conferences, theses, government reports. Academic databases such as Google Scholar, PubMed, Scopus, Web of Science and digital libraries (JSTOR, Project MUSE) were used to access scientific articles and books. Each source was carefully analyzed, evaluating

the arguments, the methods used, the results and the conclusions.
Using multiple bibliographic sources in research is essential to ensure a deep and nuanced understanding of the subject. This method contributes to the development of a well-defined theoretical framework for further research and to supporting arguments with well-grounded evidence.

RESULTS AND DISCUSSIONS

The use of natural additives in broiler nutrition is an emerging trend that responds to the need to reduce the use of antibiotics and other synthetic substances in poultry farming. These additives not only improve chicken health and performance, but also have potential benefits for human health.

Probiotics and prebiotics

Probiotics are live microorganisms that, administered in adequate doses, have beneficial effects on the intestinal microflora of chickens. Common examples include bacteria in the genera *Lactobacillus* and *Bifidobacterium.* They help improve digestion and prevent colonization of the intestine by pathogens (Alagawany et al., 2018a).
Indigestible dietary fibers that selectively stimulate the growth and activity of beneficial bacteria in the intestine can be used as prebiotics. A common example is inulin, which supports the growth of good bacteria such as *Bifidobacterium* (*B. bifidum*) and *Lactobacillus* (*L. bulgaricus, L. plantarum*) (Khaksefidi & Rahimi, 2005; Smith, 2014).
Meat from birds fed probiotics and prebiotics may have a safer microbial profile, reducing the risk of contamination with pathogenic bacteria such as *Salmonella* or *Campylobacter* (Patterson & Burkholder, 2003).
Common probiotic strains for poultry are: *Lactobacillus* spp., which are lactic acid bacteria that help maintain a healthy gut environment; *Bacillus* spp., known for spore-forming abilities, allowing them to survive feed processing and gastric acid; *Bifidobacterium* spp., which improve intestinal health by maintaining a favorable gut microbiome; *Enterococcus* spp., which contributes to a healthy gut environment and inhibits pathogens; yeasts (e.g., *Saccharomyces cerevisiae*, which improves digestion and immunity (Khaksefidi & Rahimi, 2005; Bai et al., 2013).
Probiotics for broilers are typically delivered by feed or water. So, probiotics can be incorporated into the poultry feed during the manufacturing process or they are added to the drinking water, ensuring uniform distribution across the flock.
Probiotics offer a range of benefits to broiler chickens. By enhancing nutrient absorption, probiotics lead to better growth rates and improved feed efficiency (Soomro et al., 2019). Studies show that broilers supplemented with probiotics often have a lower feed conversion ratio, meaning they require less feed to gain weight (Khaksefidi & Rahimi, 2005; Rehman et al., 2020).
In broiler chickens, probiotics play a vital role in improving immune function, enhancing disease resistance, and promoting overall health and productivity (Patterson & Burkholder, 2003). Modern poultry production relies on maintaining the health of broilers to ensure optimal growth (Soomro et al., 2019), and the use of probiotics has emerged as a natural alternative to antibiotics for improving immune function and controlling infections (Al-Khalaifah, 2018). By stimulating the immune system, probiotics increase the production of antibodies and immune cells, helping broilers fight off infections and maintain good health (Rehman et al., 2020).
One of the most significant applications of probiotics in broiler production is their ability to reduce enteric infections, which are a major cause of mortality and reduced productivity in poultry. Probiotics have been shown to inhibit the growth of harmful pathogens like *Salmonella* spp., *E. coli*, and *Clostridium perfringens*, which cause diseases such as necrotic enteritis and colibacillosis in broilers (Lutful Kabir, 2009). By competing for nutrients and attachment sites in the gut, probiotics reduce the proliferation of these pathogens, thereby lowering infection rates (Abd El-Hack et al., 2020; Bai et al., 2013).
Respiratory infections, such as those caused by *Mycoplasma gallisepticum* and avian influenza, are a common problem in broiler flocks, leading to reduced growth performance and increased mortality. Probiotics can help

improve the immune response in the respiratory tract by enhancing mucosal immunity, particularly through increased IgA production. This boosts the broilers' ability to fend off respiratory pathogens, reducing the incidence of these infections (Abd El-Hack et al., 2020; Patterson & Burkholder, 2003; Smith, 2014).

In addition to directly boosting immunity, probiotics have been shown to enhance the efficacy of vaccines in broilers. Vaccination is a common practice in poultry production to protect against diseases like Newcastle disease, infectious bursal disease, and coccidiosis. Probiotics can enhance the immune response to these vaccines by stimulating higher levels of antibody production and improving the overall immune competence of the birds (Lutful Kabir, 2009). This synergistic effect can lead to better protection against infectious diseases, improving flock health and reducing the need for additional treatments (Abd El-Hack et al., 2020).

Probiotics produce antimicrobial substances like bacteriocins and organic acids, which help inhibit the growth of harmful bacteria in the gut. This is particularly important in broilers, where maintaining a healthy gut microbiota is essential for preventing the overgrowth of pathogens like *Campylobacter*, *Bacillus*, *Salmonella*, and *Clostridium*, which are not only detrimental to broiler health but also pose food safety risks for humans. By reducing pathogen levels in the gut, probiotics contribute to both the health of the broilers and the safety of the poultry products (Anthony et al., 2009).

Probiotic supplementation has been shown to improve the structure of the intestinal villi, leading to better nutrient absorption and overall gut health (Bai et al., 2013).

Prebiotics are non-digestible food ingredients that promote the growth and activity of beneficial microorganisms in the digestive tract, particularly in the gut. In broiler chickens, prebiotics play a crucial role in maintaining gut health, supporting immune function, and improving overall growth and productivity. They serve as a natural alternative to antibiotic growth promoters, contributing to more sustainable and healthier poultry production systems (Al-Khalaifah, 2018).

By promoting the growth of beneficial bacteria and enhancing gut barrier integrity, prebiotics significantly improve gut health in broilers (Smith, 2014).

Prebiotics help to boost the immune system in broilers by increasing the production of protective antibodies and enhancing the activity of immune cells. This improved immune response makes broilers more resistant to infections and reduces the need for antibiotic treatments (Al-Khalaifah, 2018). Healthier birds are more capable of fighting off common pathogens that can compromise productivity, leading to reduced mortality and lower incidence of diseases (Windisch et al., 2008).

One of the most significant benefits of prebiotics is their ability to reduce the prevalence of gastrointestinal infections, such as necrotic enteritis caused by *Clostridium perfringens*, salmonellosis, and colibacillosis. Prebiotics help to control these infections by limiting the growth of pathogenic bacteria and enhancing the bird's immune defences, thereby contributing to healthier flocks (Patterson & Burkholder, 2003; Teng & Kim, 2018).

Studies have shown that broilers supplemented with prebiotics tend to have higher body weights, better feed conversion ratios, and lower mortality rates compared to birds not receiving prebiotic supplementation. This improved performance translates into higher productivity and profitability for poultry farmers (Ricke et al., 2020; Windisch et al., 2008).

Mannan-Oligosaccharides (MOS) is one of the most widely used prebiotics in poultry production. MOS has been shown to improve gut health by preventing the attachment of pathogens, enhancing immune function, and promoting the growth of beneficial bacteria (Patterson & Burkholder, 2003; Ricke et al., 2020).

Fructo-Oligosaccharides (FOS) stimulates the growth of beneficial bacteria, particularly *Bifidobacterium* and *Lactobacillus*, helping to improve digestion and nutrient absorption in broilers. FOS also helps reduce the population of harmful bacteria by lowering the pH in the gut (Ricke et al., 2020).

Inulin is another prebiotic fiber that promotes the growth of beneficial gut bacteria and is also associated with better nutrient absorption and improved feed efficiency in broilers (Teng & Kim, 2018).

Beta-Glucans are known for their immune-stimulating properties. In broilers, beta-glucans enhance the immune response by activating macrophages and other immune cells, helping the birds to fight off infections more effectively (Ricke et al., 2020).

Phytobiotics

Phytobiotics, also known as phytogenic feed additives, are plant-derived compounds added to animal feed to improve health, growth, and overall performance. In the poultry industry, especially for broilers, phytobiotics are gaining popularity as a natural alternative to antibiotics, which are increasingly restricted due to concerns about antibiotic resistance. Phytobiotics include a wide range of substances such as herbs, spices, essential oils, and plant extracts (Kikusato, 2021).

Commonly used herbs include oregano, thyme, rosemary, and turmeric. These herbs contain bioactive compounds that have antimicrobial, antioxidant, and anti-inflammatory properties.

Derived from plants like garlic, eucalyptus, rosemary and peppermint, essential oils have potent antimicrobial effects and promote gut health by balancing the gut microbiota (Ghazalah & Ali, 2008; Pandey et al., 2019).

The plant extracts are rich in bioactive compounds like flavonoids, alkaloids, and saponins, which have multiple benefits including enhancing immune response and improving digestion.

Numerous studies have shown that broilers fed diets supplemented with phytobiotics (rosemary essential oil and eucalyptus essential oil) have better growth rates and improved feed conversion efficiency compared to those on standard diets (Ghazalah & Ali, 2008; Mohebodini et al., 2021).

Phytobiotics can improve the sensory qualities of meat, including flavor and texture. Additionally, their antioxidant properties help extend meat shelf life by reducing lipid oxidation (Pateiro et al., 2021).

By promoting gut health and boosting the immune system, phytobiotics lower the incidence of common diseases such as coccidiosis and necrotic enteritis (Grashorn, 2010; Iqbal et al., 2020).

Since phytobiotics improve feed efficiency, they reduce the overall feed consumption required for growth, thus minimizing the environmental impact of broiler production (Mohebodini et al., 2021). Additionally, their use leads to reduced ammonia emissions from poultry waste, improving farm conditions (Ortakci et al., 2019).

Organic acids

Organic acids are a class of naturally occurring compounds used as feed additives in broiler production. They are gaining increasing attention as alternatives to antibiotics, which have been traditionally used to enhance growth and prevent diseases in poultry. Organic acids improve gut health, enhance nutrient absorption, and control pathogens, contributing to better overall performance in broilers. These compounds include short-chain fatty acids like formic, acetic, and propionic acids, as well as other organic acids like citric, lactic, and fumaric acids (Adil et al., 2010; Scicutella et al., 2021; Khan et al., 2022).

Widely used in poultry production, formic acid has strong antimicrobial properties and is effective in reducing harmful bacteria like *Salmonella* and *E. coli* in the gut. It also improves protein digestion by lowering gut pH (Aljumaah et al., 2020; Hernández et al., 2006).

Propionic acid is particularly effective against mold and fungal growth in feed, helping to preserve feed quality. In the gut, propionic acid lowers pH, improves gut health, and enhances the absorption of minerals like calcium and phosphorus (Haque et al., 2009; Moghadam et al., 2006).

Butyric acid is known for its beneficial effects on the intestinal epithelium, promoting the regeneration of gut cells and improving the barrier function of the gut. This acid plays a critical role in enhancing nutrient absorption and reducing the risk of gut-related diseases (Galli et al., 2021).

Citric acid helps in the digestion of minerals such as calcium and phosphorus by increasing their solubility. It also lowers the pH of the diet, which aids in pathogen control and enhances digestive enzyme activity (Moghadam et al., 2006; Nezhad et al., 2007; Sureshkumar et al., 2021).

Digestive enzymes

Enzymes are biological catalysts that accelerate chemical reactions, including the breakdown of complex molecules in animal diets. In broiler production, exogenous enzymes are added to feed to enhance nutrient availability and improve digestion. With modern poultry diets consisting largely of plant-based ingredients like corn, soy, and wheat, many of the nutrients are bound in complex forms that broilers' natural digestive enzymes cannot fully break down. The use of supplemental enzymes allows broilers to efficiently utilize these nutrients, leading to better growth, feed conversion, and overall health (Badshah et al., 2023; Zhu et al., 2014).

Several classes of exogenous enzymes are commonly used in broiler feed to target specific nutritional challenges.

Phytase breaks down phytate, the primary form in which phosphorus is stored in plant-based feed ingredients like cereals and oilseeds. Phytate-bound phosphorus is poorly available to broilers, but phytase liberates this phosphorus, making it digestible and absorbable. Phytase enhances phosphorus absorption, reduces the need for expensive inorganic phosphorus supplements, and decreases phosphorus excretion into the environment, reducing pollution (Alshamiri et al., 2021; Badshah et al., 2023).

Proteases help digest proteins by breaking them into smaller peptides and amino acids. Broilers may struggle to fully digest plant-based proteins like soy due to anti-nutritional factors. Protease improves protein digestion, increases the availability of essential amino acids, and enhances growth performance, while reducing nitrogen excretion, which is beneficial for the environment (Zhu et al., 2014).

Carbohydrases (xylanase, glucanase, amylase). Broilers naturally lack sufficient enzymes to digest non-starch polysaccharides, which can cause gut viscosity, impair nutrient absorption, and slow growth. Carbohydrases reduce gut viscosity, enhance nutrient absorption, improve feed conversion ratio, and boost energy availability from fibrous ingredients (Alagawany et al., 2018b; Walsh et al., 1993).

Lipases are enzymes that break down fats lipids into fatty acids and glycerol, aiding in fat digestion. They improve the digestibility of fats, allowing for better energy utilization, and contribute to optimal growth and body condition (Badshah et al., 2023).

CONCLUSIONS

Natural feed additives offer a sustainable and effective approach to enhancing broiler health, growth, and performance. As the poultry industry moves towards more natural, antibiotic-free production systems, the use of these additives will play an increasingly important role in meeting consumer demand for healthier and more sustainable poultry products.

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EFFECTS OF DIETARY OAK BARN ON PERFORMANCE TRAITS AND NITROGEN BALANCE IN LAYING HENS

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Abstract

This study aimed to evaluate the effectiveness of oak barn as a natural source of tannins included in low protein diets on the performance parameters, excreta composition, and nitrogen balance of laying hens. A total of 168 Lohmann Brown laying hens, 51 weeks of age, were divided into three dietary treatment groups and housed in digestibility cages. Laying hens were fed 17.5% crude protein (CP) in control 1 (C1), 15.5% CP in control 2 (C2), and 15.5% CP supplemented with 0.5 % oak barn in the experimental group (E). Dietary treatments registered a significant increase (p = 0.001) in laying production in the C2 and E groups, and a significant decrease in average egg weight (p = 0.0002) in the E group. The nitrogen balance assessment showed a significant decrease (p = 0.0004) of the nitrogen excreta in groups with reduced dietary protein (C2 and E) compared to control (C1). The cumulative effect of the two studied factors (oak bark and protein level) led to a reduction in nitrogen elimination through droppings of almost 33%, on average.

Key words*: nitrogen excreta, pollution, poultry, reduced dietary protein, tannins.*

INTRODUCTION

Creating a sustainable and competitive egg-production system presents a significant challenge for the laying-hen industry (Gautron et al., 2021). Although poultry production isn't a primary source of harmful gases, the excretion of nitrogen in intensive poultry production is responsible for concerning environmental issues (Gržinić et al., 2023). Additional focus is required to develop nutritional solutions for reducing nitrogen excretion and mitigating the emissions of noxious and greenhouse gases (Malomo et al., 2018). The protein content in feed shows a strong correlation with nitrogen emissions in excreta (Heo et al., 2023). Consequently, low-protein diets are widely recognized as a nutritional strategy to curb ammonia emissions while cutting down feed expenses by diminishing the need for high-cost protein sources in the overall diet (Such et al., 2021). Moreover, enhancing the digestibility of dietary protein proves to be an effective strategy for reducing nitrogen excretion (Dukhta & Halas, 2023).

Tannins, defined as a heterogeneous group of polyphenolic compounds, are secondary metabolites produced by plants as a nonspecific defense mechanism (Choi & Kim, 2020). Tannins are found in different parts of plants, like seeds, bark, wood leaves, and fruit skins, and exhibit a wide range of biological properties, such as antimicrobial, anti-parasitic, antiviral, antioxidant, anti-inflammatory, and immunomodulatory effects (Huang et al., 2018). They are primarily categorized into three main groups: hydrolyzable tannins, condensed tannins, and phlorotannins (Englmaierová et al., 2022). The condensed tannins have a higher molecular weight and more complex structure compared to hydrolyzable tannins, which could suggest a reduced bioavailability within the gastrointestinal tract of poultry (Serrano et al., 2009). Tannins have been considered antinutrients due to their association with various negative effects, such as decreased feed conversion reduced bioavailability of micronutrients, and reduced growth, these effects being particularly attributed to condensed tannins (Selle et al., 2010). Some authors suggested that a low concentration of tannins can improve feed palatability and increase the performance of monogastrics by stimulating consumption (Windisch & Kroismayr, 2006). Other authors reported that stimulation of digestive secretions may be the main mechanism of action for tannins (Li et al., 2020).

Quercus robur L., known as the common oak, is a well-known species of tree, spontaneously found in deciduous forests and utilized both in the wood industry and traditional folk medicine practices for the treatment of wounds and skin diseases (Dróżdż & Pyrzynska, 2018). In the wood industry, the bark is often deemed as waste material (Elansary et al., 2019). Oak bark comprises various polyphenols, including vanillic acid, ellagic acid, and gallic acid (De Simon et al., 1996).

Oak bark extract has been used as an anti-inflammatory agent, due to the elevated content of tannins, pectins, and flavonoids, which exhibit antimicrobial properties. Oak bark supplementation into broilers' diets resulted in increased feed intake without adverse effects on body weight (Bagno et al., 2018). Furthermore, supplementation of oak bark extracts along with enzyme preparations enhanced digestion. The inclusion of *Quercus* cortex extract, which contains biologically active compounds, into the ration of broiler chickens enhances productivity and strengthens the immune-modulating body state (Fisinin et al., 2018). Duskaev et al. (2021) showed that the addition of oak bark extract in diets can stimulate the accumulation of essential and nonessential amino acids mainly in the pectoral muscles of broilers, having a great economic value. Moreover, it was suggested that oak bark as a phytogenic feed additive can increase feed digestibility, stimulate growth, improve feed conversion ratio, and increase feed intake in broilers. Bagirov et al. (2018) reported that dietary *Quercus* cortex extract can enhance the slaughter indices of broilers, modify the fatty acid profile and the mineral composition of muscle, and impact the microbiome of the small intestine.

The goal of this study was to assess the effect of the oak barn as a natural source of tannins included in low protein diets on the performance parameters, excreta composition, and nitrogen balance of laying hens.

MATERIALS AND METHODS

The experimental procedures were conducted with approval from the Ethics Committee of the National Research and Development Institute of Animal Biology and Nutrition, Romania. These procedures adhered to the guidelines outlined in Romanian Law 43/2014 for the handling and protection of animals utilized for experimental purposes, as well as Directive 2010/63/EU concerning the protection of animals used for scientific purposes.

Experimental design

A six weeks experiment was conducted on 168 Lohmann Brown laying hens, 51 weeks old, which were assigned into 3 groups and housed in metabolic cages (Zucammi batteries Z.M.E.C 50-model 2012) that allow accurate weighing of the daily ingesta and excreta, and controlled environmental conditions. During the experimental period laying hens received the following experimental diets: a control diet (C1) containing a corn–soybean diet, a control diet with low-protein content (C2), and an experimental diet (E) with the same structure as the C2 diet and with an additional 0.5% oak bark supplement (Table 1). The oak bark used in the experiment was purchased from a local producer, and then dried, grounded, and packed. Feed and water were supplied *ad libitum*.

During the experimental period, daily records were kept for feed intake, feed conversion ratio, egg production, egg weight, and laying percentage.

During the last 5 days of the experiment, excreta samples were daily collected. Droppings samples were homogenized, dried in the drying oven BMT model Ecocell Blueline Comfort (Nuremberg, Germany) for 48 h, at 65°C and ground with Grindomix GM 200 mill (Retsch, Germany).

Chemical analyses

Feed samples were analyzed to assess the proximate composition as follows: dry matter (method 930.15; AOAC, 2005), crude protein with Tecator Kjeltek auto 1030 analyzer (SR

EN ISO 5983-2, 2009), ether extract with continuous extraction in a solvent, followed by fat measurement with Soxhlet after solvent removal (SR ISO 6492, 2001), and ash (method 942.05; AOAC, 2007). Excreta samples were analyzed for nitrogen assessment using the same methodology as for feed samples.

Nitrogen balance was assessed using the analytical data generated by the chemical analysis of the feeds and droppings, and the recorded data regarding daily feed intake and excreta. The coefficients of protein digestibility were determined using digestibility equations proposed by Sirirat et al. (2013).

Table 1. Diets formulation

Ingredients	C1	C2	E
Corn, %	30.11	36.35	35.85
Wheat, %	30	30	30
Soybean meal, %	21	15.22	15.22
Sunflower, %	5	5	5
Dl- methionine, %	0.1	0.16	0.16
L-lysine	-	0.16	0.16
L-threonine	-	0.01	0.01
Calcium carbonate, %	9.65	9.67	9.67
Monocalcium phosphate, %	0.73	0.78	0.78
Salt, %	0.37	0.37	0.37
Vegetable oil, %	1.98	1.22	1.22
Choline, %	0.05	0.05	0.05
Premix, %	1	1	1
Bark oak, %	-	-	0.5
Phytase, %	-	0.01	0.01
Total	100	100	100
Chemical analysis (calculated values)			
Metabolizable energy, (kcal/kg)	2720	2720	2720
Dry matter, %	88.78	88.72	88.72
Crude protein, %	17.5	15.5	15.5
Lysine, %	0.84	0.81	0.81
Methionine, %	0.39	0.42	0.42
Met+cist, %	0.7	0.7	0.7
Treonina, %	0.64	0.57	0.57

Statistical Analysis

The analytical data were subjected to statistical analysis through a two-way ANOVA, followed by Tukey's test, using XLSTAT software (Addinsoft, France) and Prism-GraphPad software v. 9.1.2 (San Diego, CA, USA). A probability level below 5% was considered significant.

RESULTS AND DISCUSSIONS

The effects of dietary treatments on the productive performances of laying hens are presented in Figure 1. Feeding low protein diets slightly decreased feed conversion ratio and average egg weight but the differences were not significant. Supplementation with oak bark of low protein diets led to a significant ($p < 0.001$) reduction of daily feed consumption and average egg weight, compared to the control group, while feed conversion ratio and laying intensity were not influenced by the dietary treatments.

Results of this study showed that average egg weight significantly decreased in the groups with reduced dietary protein. Similar to our results, Heo et al. (2023) reported a significant decreasing effect of weights of eggs as the reducing content of crude protein from diets, although there was a linear decrease ($P < 0.05$) in nitrogen excretion observed as dietary crude protein levels decreased, both in pullets and laying hens. This finding aligns with previous research indicating that reducing crude protein levels in diets results in decreased egg weight (Alagawany et al., 2020; Summers & Leeson, 1994). Ji et al. (2014) also noted that reduced protein concentrations did not impact growth performance and egg production, being expected that decreasing crude protein levels in chicken diets would serve as an efficient dietary strategy to mitigate the environmental impact of nutrient overload.

The chemical composition of the excreta samples was analyzed and presented in Table 2.

Table 2. Chemical analysis of laying hens' excreta

Items	C1	C2	E	SEM	P value
DM, %	25.58 ^{a}	24.44 ^{a}	23.42 ^{a}	0.532	0.262
CP, %	9.19 ^{a}	8.09 ^{a}	8.29 ^{a}	0.208	0.059
EE, %	0.52 ^{a}	0.73 ^{b}	0.61 ^{ab}	0.028	0.005
CF, %	3.44 ^{a}	3.54 ^{a}	3.35 ^{a}	0.065	0.503
CEN, %	5.76 ^{a}	5.82 ^{a}	5.59 ^{a}	0.239	0.504

Values with different superscripts in the same row are significantly different ($P < 0.05$).

Similar to the effects on production parameters, the dry matter content of the excreta was not influenced by the dietary treatments. As expected, feeding low protein diets led to a reduction in the crude protein concentrations in excreta samples from C2 and E groups, compared to the C1 group, but the differences were not statistically significant. Contrary, crude fat concentrations from excreta samples increased significantly ($p < 0.05$) in the group with reduced dietary protein levels without oak bark supplementation (C2).

Adding a source of tannins (oak bark) in the reduced protein diet (E) also led to a slight increase in crude fat concentrations from excreta, but not significantly different, compared to the control group (C1).

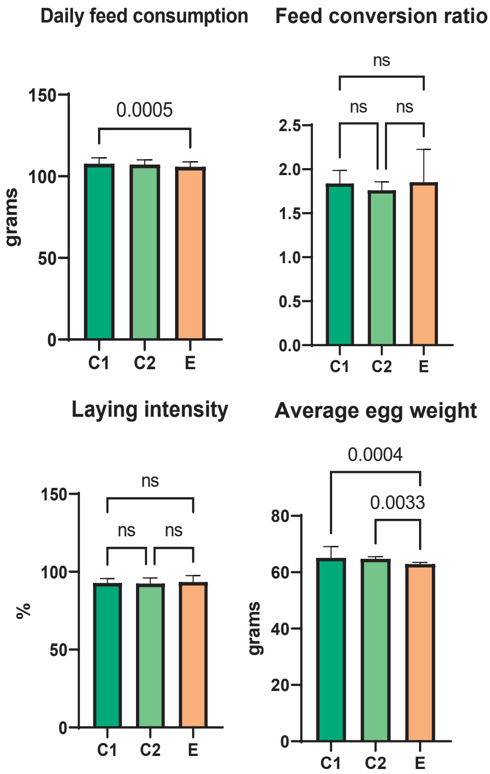

Figure 1. The influence of dietary oak bark supplementation and protein level on productive performances

The excreta collected during the balance period contained significantly ($p < 0.05$) lower amounts of nitrogen in groups with low protein diets (C2 and E), compared to the control group (Table 3). The concentrations of nitrogen decreased by 21.7 % in the group with reduced dietary content of crude protein (C2) and by 32.6 % in the group with low protein diet and dietary oak bark supplementation (E), compared to the control group (C1) (Figure 2). Ammonia emissions from poultry production represent a significant concern, necessitating the implementation of the best available practices to mitigate them. Nutrition plays a pivotal role, with feeding low-protein diets emerging as one of the most effective strategies for reducing emissions. The primary objective is to determine the optimal protein reduction level that maintains production traits without adverse effects and potentially reduces feeding costs (Belloir et al., 2015).

When employing low-protein diets, a reduction of 1% in protein content can lead to approximately a 10% decrease in nitrogen excretion (Santonja et al., 2017). Nitrogen found in droppings of laying hens originates from a mixture of undigested dietary crude protein, basal endogenous losses, and microbial fermentation by-products (Soomro et al., 2018). It is documented that excreted nitrogen can contribute to issues like eutrophication, nitrous oxide emissions, and global warming (Aneja et al., 2006). Hence, decreasing nitrogen elimination is crucial for fostering sustainable production. Implementing reduced protein diets remains a significant approach for minimizing nitrogen elimination in droppings. In a study conducted by Kerr et al. (1995), which involved the use of diets with reduced protein content and with supplementation of amino acids for poultry, a 1% decrease in dietary protein resulted in an 8.5% reduction in nitrogen excretion, irrespective of breed or body weight.

In this study, a reduction of crude protein by 2 % from laying hens' diets was applied, resulting in a 21.7 % decrease in the nitrogen excreta (C2 group) compared to the control group. The results of this research are in line with previous studies reporting that nitrogen excretion decreased linearly as the dietary crude protein levels decreased (Soares et al., 2019; Alfonso-Avila et al., 2022). The reduction in nitrogen excretion associated with decreased crude protein concentrations in diets can be associated with reduced nitrogen intake. Such et al. (2021) showed that a reduction of 2% in protein content in grower and finisher broiler diets had no adverse effects on production traits, but significantly decreased the nitrogen excretion of the birds. The same authors reported that feeding low-protein diets led to greater reductions in nitrogen excretion in younger birds. A 1% reduction in protein content could decrease nitrogen excretion by as much as 17.6% in 24-day-old birds and by 11.5% in 40-day-old animals.

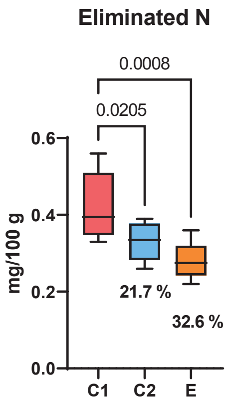

Figure 2. The influence of dietary oak bark supplementation and protein level on nitrogen excretion

Protein digestibility and nitrogen balance were assessed based on the values of feed consumption and eliminated amounts, along with the chemical composition of feeds and droppings. Protein digestibility tended to decrease in the group with reduced protein (C2) and increased in the group with supplemented oak bark in the reduced protein diet (E), compared to control. However, statistical differences were not observed in protein digestibility between the groups.

Table 3. The influence of dietary oak bark supplementation and protein level on crude protein digestibility and nitrogen balance

Items	C1	C2	E	SEM	P value
Excreted N, mg/100 g	0.422 b	0.331 a	0.285 a	0.017	0.0004
Absorbed N, mg/100 g	2.666 b	1.990 a	2.119 a	0.095	0.001
N digestibility, %	86.64 a	86.45 a	88.22 a	0.368	0.116
Excreted protein, %	2.632 b	2.064 a	1.776 a	0.103	0.0005
Absorbed protein, %	16.57 b	12.42 a	13.22 a	0.541	0.002
Protein digestibility, %	86.18 a	85.53 a	88.10 a	0.444	0.101

Values with different superscripts in the same row are significantly different ($P < 0.05$).

Prebiotics have the potential to alter gut microflora, influencing nitrogen metabolism, the quantity excreted, the ratio of fecal to urinary nitrogen, and the manner of ammonia emission from chicken manure (Such et al., 2023). Numerous tannins are regarded as sustainable feed additives because they originate from byproducts of plant-based agriculture and industry. For instance, chestnut tannins, which may be added to poultry feed, are extracted through the distillation of wood used in the construction industry (Mannelli et al., 2019).

While the inclusion of tannins in the feed of monogastric animals has been discouraged in the past due to their antinutrient properties, recent research indicates that with careful use, tannins can offer benefits to monogastric animals (Hassan et al., 2020). Smeriglio et al. (2017) suggested that the oak bark additive primarily consists of hydrolyzable tannins, with a minimal proportion of condensed tannins.

Condensed tannins have been recognized as inhibitors of various digestive enzymes, such as amylases, cellulases, pectinases, lipases, and proteases (Bhat et al., 2013). Their significant antinutritive impact can adversely affect the digestibility of lipids, starch, and amino acids (Garcia et al., 2004; Brestenský et al., 2012). Hammond et al. (2019) suggested that 3% oak bark powder is an appropriate content in poultry diets to enhance the birds' immunity and overall health without adverse effects.

Additionally, tannins have the potential to reduce the risk of livestock diseases and the spread of zoonotic pathogens. Furthermore, enhancement in performance may be a result of the beneficial influence of fostering a healthy intestinal ecosystem (Huang et al., 2018).

The mechanism by which tannins enhance growth in monogastric animals is not as well understood as it is in ruminants. One prevailing theory suggests that including tannins in low concentrations can boost feed intake and consequently improve the performance of monogastric animals (Huang et al., 2018). Moreover, while poultry possesses a distinct gastrointestinal tract compared to ruminants, tannins have the potential to modify gut health and microbiota in chickens, potentially enhancing nitrogen utilization and thereby reducing nitrogen emissions (Choi & Kim, 2020). In this study, the supplementation of a low-protein diet with oak bark led to a significant reduction of nitrogen excretion compared to the control and also a reduction of nitrogen by 14 % compared to the group with low dietary protein without supplementation with oak bark. This fact might suggest a reduction of nitrogen excretion due to the use of low-protein diets, and also a better utilization of dietary nitrogen due to the supplementation of diets with oak bark, as a source of tannins.

CONCLUSIONS

Low-protein diets can be considered one of the most effective strategies for reducing nitrogen excreta in laying hens. Moreover, enhancing the digestibility of dietary protein proves to be an effective approach in decreasing nitrogen excretion, for fostering sustainable poultry production. Results of this study showed that average egg weight significantly decreased in the groups with reduced dietary protein. The concentrations of nitrogen decreased by 21.7% in the group with a reduced dietary content of crude protein and 32.6 % in the group with a low protein diet and dietary oak bark supplementation, compared to the control group. This fact might suggest a reduction of nitrogen excretion due to the use of low-protein diets, and also a better utilization of dietary nitrogen due to the supplementation of diets with oak bark, as a source of tannins.

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CONTEMPORARY PERSPECTIVES ON THE COMPOSITION OF MILK FATTY ACIDS AND IMPLICATIONS FOR HUMAN HEALTH

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Abstract

In the past few years, there has been a notable rise in global demand for milk and dairy products. Milk fat represents an important component of milk, playing a crucial role in energy provision and contributing to numerous physical characteristics and manufacturing qualities of both milk and dairy products. The fatty acids found in milk fat are regarded as important nutritional components in the human diet. Generating milk with an increased concentration of polyunsaturated fatty acids (PUFAs), particularly from the n3 category, is advantageous because dietary patterns containing more n3 fatty acids and fewer n6 fatty acids are considered healthier for humans. There are numerous factors that influence the fatty acid in the milk profile, with nutrition being the most critical aspect among them. Primary dietary factors, including the type and quantity of either forages or concentrates within the dietary regimen, extensive research has been conducted on the balance between forage and concentrate ratios, as well as the inclusion of fat or oil supplements in diets.

Key words: *alternative food, dairy cattle, fatty acids, feeding, health.*

INTRODUCTION

In recent times, there has been a worldwide inclination towards a heightened request for milk and dairy items (OECD/FAO, 2020; EC, 2020). Milk fat represents a significant constituent of milk, playing a pivotal role in energy provision and contributing to numerous physical attributes and manufacturing qualities of both milk and dairy products (Bauman & Griinari, 2001; Alatas et al., 2015).
Fatty acids (FAs) present in milk fat are deemed significant nutritional elements in the human diet (Hanus et al., 2018). From a nutritional standpoint, it is desirable to produce dairy with a larger amount of polyunsaturated fatty acids (PUFAs), particularly those from the n3 group, because diets containing more n3 and less n6 fatty acids are considered healthier for humans (Simopoulos, 2002; Moallem, 2018). Fatty acids can be classified into several categories, including saturated, monounsaturated, polyunsaturated, and acetylenic fatty acids. Additionally, there are fatty acids with unique structural features, such as branched, cyclic, epoxy, hydroxyl, and those containing a ketone group (Pece et al., 2007; Danila et al., 2022).
There are numerous factors that impact the fatty acid (FA) composition of milk, nutrition playing a pivotal role. Key dietary factors, such as the type and amount of forages or concentrates consumed, the balance between forages and concentrates, and the incorporation of fat or oil supplements into diets, have been thoroughly investigated (Hanus et al., 2018).

MATERIALS AND METHODS

For the completion of this study, a systematic search of other relevant scientific articles on the chosen subject was conducted in the Google Scholar, PubMed, and MDPI databases. Relevant articles published in recent years were examined, and the references of included studies were also consulted to obtain additional bibliographic sources.

RESULTS AND DISCUSSIONS

Exploring the role of milk fatty acids in nutrition and human health

Fatty acids found in milk fat are crucial nutritional constituents in the diets of many people, with significant implications for human health. While historically associated with adverse effects, recent scientific understanding has led to a more favorable perception of milk fat's influence on human health (Nicolosi et al., 1997; Parodi, 1997; Parodi, 1999; Dhiman et al., 2005; German et al., 2009; Parodi, 2009; Chung et al., 2018; Hadrova et al., 2021).

Historically, there has been an association between fatty acids and adverse health effects. However, over the past decade, this perception has been significantly reassessed.

Milk fat contains numerous compounds with potential anticarcinogenic properties, including conjugated linoleic acid, sphingomyelin, other sphingolipids, butyric acid, 13-methyltetradecanoic acid, ether lipids, as well as vitamins A and D. Research suggests that milk fat provides protection against various types of cancer, including breast, skin, stomach, prostate, and colon cancer (Parody, 2004; Danila et al., 2022).

Dairy products serve as significant sources of conjugated linoleic acid (CLA), particularly the cis-9, trans-11 isomer. The level of CLA found in milk fat typically ranges from 3 to 5 mg/g, with the cis-9, trans-11 CLA isomer comprising approximately 80-90% of this concentration (Parody, 1997; Sehat et al., 1998; Danila et al., 2022). The dietary composition of ruminants, particularly dairy cows, significantly influences alterations in milk FAs, indicating the potential for targeted dietary adjustments to yield milk with a favorable fatty acid profile, aligning with human nutritional guidelines (Shingfield et al., 2008).

Generally, there is a health-oriented preference for increasing the levels of n3 fatty acids in milk and dairy products, while concurrently reducing the presence of specific saturated fatty acids (SFAs) due to their association with heightened atherosclerotic risks (Lock & Garnsworthy, 2001; Socha et al., 2007). The content of PUFA and SFA in milk fat can be altered by adjusting the proportion of fresh grass, hay and maize silage in the dietary intake.

Additionally, the consumption of fresh grass boosts the level of CLA, a biologically active compound known for its anticarcinogenic effects (Ip et al., 2003; Bargo et al., 2006). The reaction in milk production and composition exhibits more variability when fat supplements are added to the diet compared to diets based on corn silage as forage. Conversely, diets with higher concentrate contents can potentially cause milk fat depression and alterations in the milk fatty acid profile (Reno et al., 2013; Liu et al., 2008; Bauman & Griinari, 2003). Supplementation of dairy cow diets with linseed oil, sunflower oil, or fish oil can influence the PUFA content in milk, particularly ALA and CLA isomers. Controlling the ruminal biohydrogenation process can enhance improving the health profile of cow milk by augmenting the levels of CLA and n-3 fatty acids (Mach et al., 2013; AbuGhazaleh, 2008; Thanh & Suksombat, 2015; Silvia et al., 2015).

Dairy cows primarily acquire fatty acids for milk fat synthesis from various sources, including the diet itself, rumen microorganisms, adipose tissues, and biosynthesis occurring anew in the mammary gland (Walker et al., 2004). The proportion of fatty acids from each source in milk fat production varies significantly based on factors such as feed intake, diet composition, and lactation stage (Shingfield et al., 2008). Increased starch intake is linked to enhanced de novo synthesis, resulting in a higher proportion of saturated milk fat, whereas higher intake of polyunsaturated fatty acids from pasture leads to elevated concentrations of specific fatty acids in milk fat (Parodi, 2009).

Milk fat content typically rises with the increasing fiber content of various forages. Incorporating forage, especially fresh grass, increases the ratio of unsaturated fatty acids to saturated fatty acids in cow milk fat (Bauman & Griinari, 2001; Lock & Garnsworthy, 2001; Elgersma et al., 2013; Dewhurst et al., 2006). Additionally, the intake of fresh grass elevates the concentration of specific fatty acids with beneficial effects on human health (Parodi, 2009). When fat supplements are introduced into the diet, the reaction in both milk production and composition varies in comparison to diets primarily reliant on corn silage as the main forage source. Diets with higher concentrate contents can potentially cause alterations in the milk fatty acid profile, highlighting the importance of balanced dietary compositions (Bauman & Griinari, 2001; Lock & Garnsworthy, 2001). Supplementation with linseed oil, sunflower, and fish oil can impact the fatty acid composition of milk, presenting opportunities to enhance its healthiness

(Shingfield et al., 2008; Parodi, 2009). Fish oil, rich in EPA and DHA, modulates the biohydrogenation process, influencing the synthesis of specific fatty acid isomers with potential health benefits (Simopoulos, 2002). The consumption of certain fatty acids has been associated with several health advantages, such as lowered cardiovascular risks and enhanced neurological function (Rizos et al., 2012; Simopoulos, 2002). Continued research in this field is essential to comprehend fully the effects of animal diets on milk composition and human health (Griinari & Bauman, 1999). This could lead to the development of more effective dietary strategies and the promotion of healthier lifestyles (Parodi, 2009; Rizos et al., 2012). Dairy products with an optimal fatty acid composition could positively impact consumer health and well-being (Simopoulos, 2002).

Alternative feeding resources for dairy cows

When it comes to dietary characteristics, substituting forage with oilseed brings about notable shifts in certain fatty acid (FA) profile outcomes. Meta-regression findings indicate that as the difference in forage inclusion increases (supplemented minus control values), there's a reduction in CLA content ($\beta = -0.09$; $p < 0.05$) and Σ omega-6 ($\beta = -0.11$; $p < 0.05$). Moreover, there's an observed increase in unsaturated fatty acid (UFA) milk content with a rise in NDF difference ($\beta = 0.19$; $p < 0.001$). Elevated levels of linoleic acid (LA) in the supplemented group are significantly associated with decreased values of lactose ($\beta = 0.005$; $p < 0.001$), linolenic acid (LNA) ($\beta = -0.13$; $p < 0.001$), oleic acid (OA) ($\beta = -0.23$; $p < 0.001$), and vaccenic acid (VA) in milk ($\beta = -0.04$; $p < 0.05$). Additionally, there's a negative correlation between the difference in LNA and OA effect size ($\beta = -0.15$; $p < 0.001$) (Vargas-Bello-Perez et al., 2020).

Incorporating oilseeds into the diet doesn't impact remains unaffected by the isoenergetic balance between diets observed in most trials, thus not influencing the productive behavior of ruminants (Vargas-Bello-Perez et al., 2020). Similarly, Rabiee et al. in 2012 didn't find a statistical difference when using oilseeds; nevertheless, it can improve milk production with the utilization of an alternative lipid source, where the variation is linked to the dry matter intake and energy content of the diet.

Sunflower, cottonseed, and soybeans significantly contribute to a decrease in milk fat, possibly linked to the quantity of LA (greater than 50 g 100 g^{-1} FA), although this remains unclear. Nevertheless, LA is believed to serve as a substrate in the formation of trans isomers, which have been associated with milk fat depression syndrome. Conversely, supplementation with flaxseed and rapeseed has a has a lesser impact on LA content, possibly due to saturation by alternative pathways of their main fatty acids, resulting in fewer intermediate isomers inhibiting de novo synthesis of FA in milk fat (Bauman & Griinari, 2003; Leduc et al., 2017).

Oilseed use in diets for ruminants can elevate propionic acid production and decrease acetic acid production proportionally. This occurs as a result of glycerol release from triglycerides during lipolysis. At this shift in the rumen, the ratio of acetic to propionic acids does not impact milk yield but promotes conditions (primarily rumen pH and/or microbial population) for increased unsaturated fatty acid (UFA) content. Long-chain polyunsaturated fatty acids (PUFA) escaping biohydrogenation are absorbed by the intestine, increasing the UFA ratio in milk at the expense of saturated fatty acids, possibly due to an inhibitory effect of acetyl-CoA carboxylase and fatty acid synthetase enzymes at the mammary gland level (Martinez et al., 2013; Kholif et al., 2018; Pi et al., 2019; Vargas-Bello-Perez et al., 2020).

The significance of enhancing the PUFA proportion in milk lies in its potential to decrease obesity and mortality from cardiovascular issues by as much as 30% and decrease diabetes incidence by up to 50%. Omega-3 PUFA exhibited a 20% decrease in mortality among patients with cardiac issues (Livingstone et al., 2013; Maki et al., 2018).

The abundant presence of linoleic acid (LA) in sunflower seeds, comprising 60.4% of fatty acids (FA), has a significant impact on the fat content of milk. Introducing polyunsaturated fatty acid (PUFA)-rich sources as supplements leads to a reduction in acetic and butyric fermentation in the rumen, thereby leading to a reduction in the synthesis of new fats within the mammary gland and the inhibition of lipogenic enzymes (Ueda et al., 2003). In contrast, the incorporation of sunflower into the diets of cows has a beneficial effect on the content of oleic acid (OA), likely attributable to the potential escape of fatty acids from ruminal biohydrogenation and their subsequent absorption by the small intestine for secretion in milk, or through the activity of the delta-9 desaturase enzyme in the mammary gland (Rabiee et al., 2012). Moreover, the inclusion of rapeseed and soybean in the rations of dairy cows shows a positive correlation with OA content, presumably due to the elevated concentration of this fatty acid in these types of oilseeds.

Higher levels of oilseed supplementation exhibit a positive linear impact on unsaturated fatty acid content when linseed, rapeseed, and soybean are utilized. The forage level in the diet strongly correlates with saturated fatty acid contents in milk. Interestingly, experimental washout periods impact unsaturated fatty acid contents, specifically rumenic (CLA), vaccenic, linolenic, and oleic fatty acids (Plata-Perez et al., 2022).

Marine algae encompass a wide array of valuable, biologically active compounds, including polysaccharides, proteins, PUFAs, various pigments, and antioxidants, divided into macroalgae and microalgae based on size. Genera and species like *Ascophyllum nodosum*, *Laminaria* sp., and others hold potential for use as animal feeds. Despite low lipid content, macroalgae are rich in PUFAs, particularly EPAs and AAs, found primarily in brown and red seaweeds. Scientific data on the effect of macroalgae supplementation in ruminant diets on milk yield and composition are limited but indicate increased milk fat yields with supplementation of certain species like *Lithothamnion calcareum*. Microalgae produce a variety of bioactive compounds, rendering them suitable supplements not just in human diets but also in animal diets. Chlorella and Spirulina, among others, are notable for their high protein content and are rich sources of various fatty acids. Their supplementation in cow diets can influence milk FA profiles, impacting rumenic acid, n3 FAs, and DHA transfer efficiency into milk fat. (Dawczynski et al., 2007; Cruywagen et al., 2015; Makkar et al., 2016; Khan et al., 2018; Neville et al., 2019; Morais et al., 2020).

Oilseeds, rich in PUFA, can be provided to dairy animals to alter milk fatty acid profiles, producing nutritionally beneficial milk. Flaxseed decreases short- and medium-chain FA concentrations while increasing long-chain FA content in milk fat. Fish oil addition has been suggested to increase long-chain n-3 FA contents in milk. Clover in organic forage may reduce rumen biohydrogenation of unsaturated FA (Chilliard et al., 2001; Dewhurst et al., 2003). Linseed supplementation elevates nutritionally desirable FA content in milk, which could be important for human nutrition (Butler et al., 2011). Although oilseed supplementation reduces SFA concentrations and increases desirable FA, it may not completely offset the absence of fresh forage in the diets of housed cows. Rapeseed is inferior to linseed in improving milk fat composition. Proposed dietary combinations can maximize positive impacts on milk FA profiles without compromising milk production and solids content (Stergiadis et al., 2014).

Linseed supplementation reduces C16:0 proportion while increasing omega-3 fatty acids and conjugated linolenic acid concentrations, improving milk quality. Additionally, linseed supplementation affects gene expression related to metabolism and immune response in the liver and mammary gland, providing insight for improving milk quality and animal health (Mach et al., 2013).

CONCLUSIONS

The understanding of the role of fatty acids found in milk fat has evolved significantly over time, with recent research highlighting their potential benefits for human health. While historically associated with adverse effects, scientific advancements have led to a more favorable perception of milk fat's influence on human well-being.

Milk fat contains compounds with potential anticarcinogenic properties, providing protection against various types of cancer. Dairy products, particularly those rich in conjugated linoleic acid (CLA), offer significant health benefits. The composition of fatty acids in milk can be influenced by dietary adjustments, with targeted strategies yielding milk with a favorable fatty acid profile. Increasing levels of certain fatty acids, such as n3 fatty acids, while reducing specific saturated fatty acids, align with health-oriented dietary preferences.

Furthermore, supplementation with oilseeds and other dietary modifications can enhance the healthiness of milk by altering its fatty acid composition. Continued research in this field is crucial for developing effective dietary strategies and promoting healthier lifestyles, as dairy products with optimal fatty acid profiles could positively impact consumer health and well-being.

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INFLUENCE OF FROZEN AND DRIED POLLEN FEEDING ON FAT BODY DEVELOPMENT IN WORKER BEES (*Apis mellifera* L.)

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Abstract

The study presents the influence of frozen and dried pollen feeding on the fat body development (FBD) of worker bees (Apis mellifera L.). The degree of FBD was determined before and after feeding during the Autumn and Spring periods. During Autumn, the highest percentage of bees (42.9%) with a second degree of FBD was found after feeding with dried pollen. During the same period, 38.1% of bees fed in this way were observed to have reached the third degree of FBD, and 19.0% of bees had reached the fourth degree of FBD. After completion of spring feeding with dried pollen, the highest percentage of bees (58.6%) with the fourth degree of FBD was reported. This percentage was significantly higher than the percentage before feeding (30.8%), with a difference of 27.8%. A significant increase in the percentage of bees (11.1%) fed with frozen pollen with the fifth degree of FBD was recorded for this period. The high degree of FBD of the worker bees indicates the greater effect of frozen pollen feeding compared to dried pollen feeding during the spring period.

Key words: *Apis mellifera L., fat body, feeding, frozen and dried pollen, honey bees.*

INTRODUCTION

The presence of pollen and honey in nature is very important for bee specimens and has a direct impact on the development and functioning of their fat body. Worker bees accumulate fat deposits, carbohydrates, and proteins in it after consuming pollen and honey. Pollen is a major source of amino acids, proteins, fats, vitamins, and minerals for honey bees (Stanley & Linskens, 1974; Roulston & Buchmann, 2000). It is a major factor determining the life span of specimens (Haydak, 1970).

According to some authors, insufficient quantity and poor quality of pollen in nature lead to delayed development of bee brood (Smart et al., 2016), suppression of bee reproduction, and the emergence of worker bees with shorter lifespans (Zheng et al., 2014). The increase and decrease in brood areas depend on the egg-laying activity of queen bees, as well as the availability of pollen in the environment during different seasons of the year (Liolios et al., 2015; Di Pasquale et al., 2016; Filipiak et al., 2017). The pollen collected by bees during autumn is crucial for bee colonies as it is rich in nutrients necessary for bees during wintering (De Grandi-Hoffman & Chen, 2015). There is evidence that "forager" bees exhibit preferences for specific micronutrients in pollen, which vary depending on the season (Bonoan et al., 2018). The rich nutrient content of pollen contributes to the development of worker bee fat bodies (Alaux et al., 2010). Most of the fatty acids obtained from food are transported to it (Skowronek et al., 2021). An unbalanced diet rich in fatty acids disrupts the ability of worker bees to recognize diseased broods, which can negatively affect bee colony hygiene (Bennett et al., 2022). According to Maurizio (1961), the degree of development of the fat body in bees is an important indicator of the physiological state of the insects. Research by several authors shows that it actively participates in the processes of metabolism of other organs as well (Hoshizaki, 2013; Aljedani, 2018). In periods of stress for bee colonies, such as a shortage of food in nature, a long flight of bees, a sudden change in environmental temperature, etc., honey bees maintain homeostasis in their body by mobilizing nutrients from their fat body with the participation of various hormones - octopamine, adipokinetic hormone, and juvenile hormone (Beenakkers et al., 1985; Gruntenko et al., 2000; Hirashima et al., 2000; Arrese & Soulages, 2010; Kodrik et al., 2015;

Tao et al., 2016). The degree of development of the fat body is also of particular importance in the production of royal jelly and the feeding of brood by young worker bees (Crailsheim, 1992). Some authors have reported that malnourished bees have less developed fat bodies (Keller et al., 2005; Toth & Robinson, 2005). Others have found that during the last 1-2 weeks of the life of worker bees, their fat body stores decrease (Toth & Robinson, 2005). Differences in the degree of development of the fat body have been observed in summer and winter bees (Lotmar, 1939). Fluri & Bogdanov (1987) proved that the fat body is larger in winter bees. The food reserves accumulated in it during the autumn are used by the bees during wintering (Maurizio, 1961). Shumkova et al. (2019) found a positive influence of stimulating feeding of bee colonies in the autumn period on the degree of fat body development of worker bees. According to several authors, the floral origin of pollen consumed by bees is also of great importance for the development of the fat body of bees (Di Pasquale et al., 2013). In beekeeping practice, the feeding of bee colonies takes place mostly in the spring and autumn periods. When there is a shortage of pollen in nature and bee nests, bee colonies are provided with protein substitutes for pollen or foods with added pollen. After collecting pollen from the bee colonies, it is stored by drying (Regulation No. 9, 2005; Dominguez-Valhondo et al., 2011) or freezing (Nath & Anderson, 1975). In this regard, it is appropriate to investigate whether there are differences regarding the development of the fat body of worker bees from colonies additionally fed with frozen and dried pollen.
The study's objective is to investigate the influence of feeding bee colonies with frozen and dried pollen on the development of the fat body in worker bees (*Apis mellifera* L.).

MATERIALS AND METHODS

The survey was conducted in the fall of 2022 and the spring of 2023 at the Educational Experimental Base of the Beekeeping section at the Faculty of Agriculture of Trakia University - Stara Zagora, Bulgaria. Feeding of bee colonies of the local honey bee (*Apis mellifera* L.) previously equalized in terms of strength, number of brood, and food reserves was carried out. Two experimental and one control groups were formed with two bee colonies each, as follows:

- **1st Experimental group** – six feedings at seven days' intervals with 160 g polyfloral bee honey mixed with 40g of dried pollen;
- **2nd Experimental group** – six feedings at seven days' intervals with 160 g polyfloral bee honey mixed with 40g of frozen fresh pollen;
- **Control** – six feedings at seven days' intervals with 200 g polyfloral bee honey.

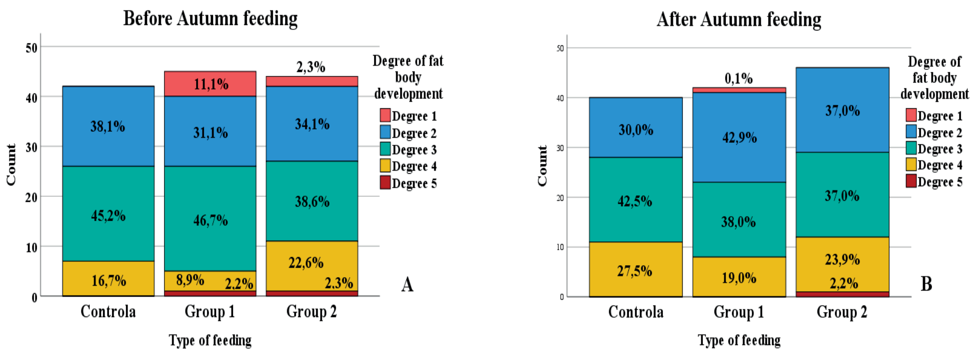
Worker bees were sampled from all bee colonies to determine the degree of fat body development before and after feeding in the autumn and spring periods. The degree of development of the fat body of 530 bees was determined according to the method of Maurizio (1954) - a 5-point scale.
Statistical software IBM® SPSS® Statistics 26.0 (NY, USA) was used for data processing, applying the non-parametric method "x^2 – analysis" (chi-square).

RESULTS AND DISCUSSIONS

1. Autumn period

Figure 1 illustrates the results of the x^2-analysis regarding the degrees of development of worker bees' fat bodies for the periods before and after autumn feeding. Before autumn feeding, the highest percentages of bees whose fat bodies reached the third degree of development have been recorded, both for the experimental groups (46.7%; 38.6%) and for the control group (45.2%). The highest percentage of bees with fat bodies that reached the fourth degree of development has been reported for Group 2 (22.6%), followed by the control group (16.7%). A probable reason for this relatively high rate of fat body development in bees is the supply of bee colonies with pollen from the summer period. The presence of bee pollen in bee colonies during this time of the year is a crucial condition for raising brood from which worker bees will hatch for the successful wintering of the colony (Dorea et al., 2010). The percentage of bees with fat bodies that reached the fifth degree of development is the same in both experimental groups. In the control group, no

bees have been found whose fat bodies have reached the fifth degree of development.
The results for the autumn period after feeding the bee colonies show that when feeding with dried pollen, the highest percentage of bees whose fat bodies have reached the second degree of development (42.9%). The third degree of development has been reached in 38% of the bees also fed with dried pollen. Interestingly, the fourth degree of development of the fat body has been observed in 19.0% of the bees from Group 1, and before feeding this percentage was only 8.9%.
As for bees fed frozen pollen, a higher percentage of fat body development (fourth degree) has also been recorded after feeding (23.9%) compared to the percentage before feeding (22.7%). An equal percentage of bees (37.0%) whose fat bodies have reached the second and third degrees of development has been reported in the group fed with frozen fresh pollen. The percentage of bees whose fat bodies have reached the fifth degree of development was small and remained relatively constant before and after autumn feeding with frozen pollen. In the control group, a high percentage of bees with the third degree of fat body development has also been reported (42.5%), followed by those with the second degree of development (30.0%). A relative increase of bees from the control group whose fat bodies have reached the fourth degree of development (27.5%) has been observed, which is most likely due to the pollen obtained from nature.



Cramer's V = 0.204; Sig. (p) = 0.026 Cramer's V = 0.117; Sig. (p) = 0.471

Figure 1. Influence of feeding on the degrees of fat body development of worker bees before and after Autumn feeding
* $p < 0.05$, Group 1 – feeding with dried pollen, Group 2 – feeding with frozen pollen

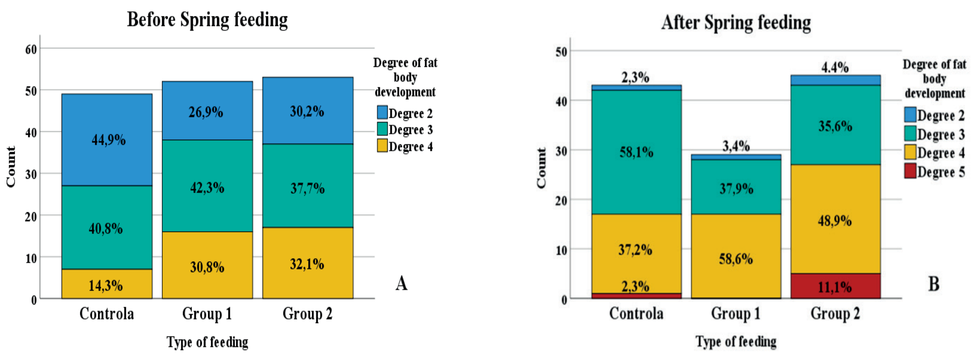
Considering the fact that all the bee colonies included in the study were under the same climatic conditions and bee pasture, it can be assumed that the differences in the development of the fat bodies of the bees from the analysed samples are to a certain extent due to the feeding as well. A support for this statement is Cramer's V coefficients (0.204 and 0.117), which show that the influence of the type of feeding on the development of fat bodies of worker bees is weak, but statistically significant.

2. Spring period

The provision of pollen in bee nests during the Spring period is essential for the proper development of bee colonies. The lack of food, especially the lack of pollen, leads to the weakening of bee colonies (Mattila & Otis, 2006). The nutrients in pollen (proteins, lipids, vitamins, and minerals) are essential for the survival of the colonies (Brodschneider & Crailsheim, 2010). According to some authors, complete deprivation of pollen may reduce the lifespan of bees (Wang et al., 2014). Some authors have reported that malnourished bees have less developed fat bodies (Keller et al., 2005; Toth & Robinson, 2005).
Figure 2 illustrates the results of the x^2-analysis regarding the degrees of development of worker bees' fat bodies for the periods before and after spring feeding. Before the spring feeding, the established trend of development of the fat bodies was preserved, as before the

autumn feeding. The highest percentages of bees whose fat bodies had reached the third degree of development have been found in the experimental groups (42.3%; 37.7%). In the control group, the percentage of bees whose fat bodies had reached the second degree of development was the highest (44.9%), followed by those with the third degree of development (40.8%). The highest percentage of bees whose fat bodies had reached the fourth degree of development has been reported for Group 2 (32.1%), followed by Group 1 (30.8%). No bees have been found the fat bodies of which had reached the fifth degree of development.
After the end of the spring feeding, it has been found that when feeding with dried pollen, the highest has been the percentage of bees the fat bodies of which had reached the fourth degree of development (58.6%). For comparison before feeding, the percentage in this group had been 30.8%. A difference of the order of 27.8% is observed, which is indicative of the effect of additional feeding. Other authors have reached similar conclusions (Shumkova et al., 2019). In the present experiment, it has been found that no bees whose fat bodies had reached the fifth degree of development when feeding with dried pollen.

In the bee colonies fed with frozen pollen, there is a tendency to increase the number of bees whose fat bodies have reached the fourth degree of development (48.9%). An even higher percentage of difference has been found compared to the percentage for this group before feeding (32.1%). The stated difference is 16.8%. In addition, a significant increase in the number of bees with fat bodies developed to the fifth degree (11.1%) has been reported. This shows the greater effect of feeding bee colonies with frozen pollen compared to dried pollen during the spring period. Although there is an influx of pollen from the flowering plant species in the environment during the spring period in the beehives, it turns out that the addition of extra amounts of pollen has a positive effect on the development of the fat bodies of worker bees. In the control group, the percentage of bees whose fat bodies had reached the third degree of development was the highest (58.1%), followed by those with the fourth degree of development (37.2%). In this group, 2.3% of bees with the fifth degree of development of their fat bodies have been found.



Cramer's V = 0,147; Sig. (p) = 0,050

Cramer's V = 0,209; Sig. (p) = 0,016

Figure 2. Influence of feeding on the degrees of fat body development of worker bees before and after Spring feeding
* $p < 0.05$, Group 1 – feeding with dried pollen, Group 2 – feeding with frozen pollen

As with autumn feeding, Cramer's coefficients (0.147; 0.209) show a weak but statistically significant relationship between the type of feeding and the development of worker bees' fat bodies. Based on the results of the spring feeding of the bee colonies with frozen and dried pollen, it can also be assumed that the differences in the development of the fat bodies of the bees from the analyzed samples are to some extent also due to the feeding. Similar conclusions have been reached by other authors (Shumkova et al., (2019).

CONCLUSIONS

In the autumn period after feeding the bee colonies, the highest percentage (42.9%) of development of fat bodies reaching the second degree was found in the bees fed with dried pollen.

During the same period, the third degree of development of the fat body was reached in 38% of the bees fed with dried pollen, and the fourth degree of development was observed in 19.0% of the bees from this group, while before feeding this percentage was only 8.9%.

After completion of spring feeding with dried bee pollen, the highest percentage of bees in which fat bodies reached the fourth degree of development (58.6%) was reported compared to the percentage before feeding (30.8%), with a significant difference in the order of 27.8%.

For this period of the year, when feeding with frozen pollen a significant increase in the percentage of bees whose fat bodies are developed to the fifth degree (11.1%) was recorded. This shows the greater effect of feeding bee colonies with frozen pollen compared to dried pollen during the spring period.

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ISOLATION OF CARBAPENEM-RESISTANT *Klebsiella pneumoniae* FROM MASTITIC COWS AND THEIR ENVIRONMENTS

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Abstract

Klebsiella species cause infections occurring in different tissues of various hosts. In terms of bovine health it is a well-known opportunistic pathogen playing a role in the pathogenesis of mastitis. In fact, such bacteria can spread widely in bovine farm environments mostly through dairy facilities and breeding areas causing eventually mastitis. Characterizing aetiological agents thoroughly can assist to understand pathogenesis of the opportunistic infections. In this study, a total of 1206 dairy cows from 6 farms were first screened by California Mastitis Test (CMT). Samples found positive by CMT, samples from clinical mastitic udders and also sıwab samples obtained from both the same animals' rectal and nasal orifices and their surrounding environments were all cultured aerobically and a complete identification of the isolates was achieved by phenotyping and genotyping. Some bovine Klebsiella strains from the culture bank of the Department were also included as organ isolates in the study. Lastly, antibiotic resistance of the strains was detected. There is no difference between numbers of coliforms from the farms using either robotic milking or classical milking systems ($p > 0.05$). The highest prevalence of Klebsiella mastitis in the farms examined in this study was 8.75%. It was common to see colistin resistance in the Klebsiella isolates from all farms anyway. The lowest 12% and highest 50% resistances for colistin were seen in rectal and organ originated strains, respectively. Unexpectedly, carbapenem (Imipenem) resistance was detected and was the highest 50% in isolates from environments. The lower occurrence of carbapenem resistance 18.2% was measured in Klebsiella spp. isolated from mastitic milk samples. Carbapenem resistancy was further verified molecularly.

Key words*: Carbapenem, dairy cow health, environmental contamination, opportunistic infections.*

INTRODUCTION

Klebsiella species are Gram-negative, rod-shaped, encapsulated, lactose positive (with an exception of the species *Klebsiella pneumoniae* subsp. *rhinoscleromatis*), non-motile, H_2S negative, facultatively anaerobic bacteriae (Atalay, 2023; Cheng et al., 2021) *Klebsiella pneumoniae* (*K. pneumoniae*) causes high morbidity rates and significant economic losses in cases of mastitis (Oliver, Gonzalez, Hogan, Jayarao, & Owens, 2004). Bovine mastitis leads to major economic losses and some profound, negative effects on animal welfare. Thus, it is important to successfully manage such infections including especially those that at the beginning or subclinical stage of the inflamemation. *K. pneumoniae* is generally considered as one of the opportunistic pathogens causing not only environment-derived bovine mastitis but also upper respiratory tract infection of dairy cattle. It is an emerging zoonotic and foodborne pathogen with a presence in many countries worldwide (Darniati et al., 2021). *K. pneumoniae* is now considered as one of the major pathogens of international concern due to the dramatic increase in the occurrence of its hypervirulent as well as carbapenem-resistant strains (Chang et al., 2021). The bacterium's widespread presence in areas heavily used by humans, such as dairy farms increases the risk of infection (Jayarao et al., 2006). Compared to other pathogenic microorganisms, *K. pneumoniae* can lead to a faster and more severe occurrence of mastitis by means of its strong host specific growth feature (Schukken et al., 2012). This often increases an urgent need for appropriate and rapid treatment (Vikova et al., 2017). However, many *K. pneumoniae* strains show resistance to different antibiotics, which limits treatment choices, and more complex treatment protocols are required (Paczosa & Mecsas, 2016; Ruegg, 2017). More researches on *Klebsiella* were rather focused on human health until recently (Chang et al., 2021;

Schukken et al., 2012). However, it can be argued that virulence genes in the human isolates show similarities to those of animal-derived strains. This suggests that the adoption of any approaches to follow up sources of infections epidemiologically may be necessary (Yang et al., 2019). More investigation is still needed on genotypic and phenotypic features that may affect the good management of bovine mastitis. Specifically, for *K. pneumoniae* to establish an udder infection, the bacteria must overcome various mechanical and chemical barriers and bypass the humoral and cellular defense system of the host (Piperaki et al., 2017). Antibiotic resistance profiles of animal originated strains are also required to be updated at all time to treat effectively patients of animal origin.

Researches on bovine mastitis caused by *Klebsiella* spp. conducted in this country so far produced little data and showed some occurrence rates. An early of these studies conducted on 1277 dairy cows from some state farms located in Bursa, Eskişehir and Ankara reported no *Klebsiella* isolation. Another study later carried out in a farm from Ankara also reported no isolation (Arda & Istanbulluoglu, 1980; Ulusoy, 1985). Among 21 other studies on bovine mastitis in Turkey covering the period from 1979 to 2022, the highest rate for *Klebsiella* spp. isolation was observed 34.3% in Aydın region (Erdoğdu, 2019). In another study from the same city 20 years later, a total of 141 milk samples taken from cows with clinical mastitis were examined and *K. pneumoniae* was detected in 5% of 141 milk samples examined (Kaya et al., 1999).

In other studies in Turkey, the isolation rates were between 0% and 17.9% depending on the year and geography of the studies as reviewed elsewhere (Atalay, 2023).

As for the studies in Konya and its district; *Klebsiella* isolation was not stated in previous reports from cow milk with mastitis (Ateş et al., 1991; Bozkır, 1985; Dinç et al., 1991; Tekeli et al., 1985). The first report ever on *Klebsiella* spp. in Konya region was made from sheep mastitic milk (Erer et al., 1990). In the following years, *Klebsiella* spp. has been beginning to be isolated from bovine mastitic milk as evidenced by a few studies (Nizamlıoğlu et al., 1992). Therefore, we aimed to detect up-to-date prevalence and profile of carbapenem-resistance in *Klebsiella* spp. isolates from the cases of bovine mastitis, Konya by this study.

MATERIALS AND METHODS

Sampling, Isolation and Identification

The study was conducted on 1206 lactating cows from 6 intensively managed farms in Konya. All the cows were examined clinically first and then those that were not showing any visible signs of mastitis were screened using California Mastitis Test (CMT). Subclinical mastitis was diagnosed based on the screening test. The CMT positive milk samples (n = 124) as well as milk samples from udders showing any signs of clinical mastitis (n = 67) were collected to the 15 mL sterile tubes and transferred to the lab in isothermal boxes 4°C for bacterial culture. The microbiological examinations were carried out at the Microbiology laboratory of the Veterinary Faculty of Selçuk. A loopful of milk sample inoculating onto Tryptic Soy Agar (TSA) enriched with 5% sheep blood (Merck 105459/Almanya) and were incubated for 24-48 hours at 37°C under aerobic conditions. Suspicious coliform isolates based on colony morphology and gram staining characteristics were then transferred to McConkey Agar (Lab M Limited/UK) and incubated at 37°C overnight. Identification of the isolates was performed using Lassen Triple Tube Method, IMVIC and additional tests (Hogan et al., 1999; Lassen, 1975; Quinn et al., 2002). Swab samples taken from nasal, rectal orifices and from environmental surfaces were taken into tubes containing Todd Hewit Broth (Neogen-NCM0061/USA) and inoculated onto the same agar as above and followed the same procedures for isolation and identification. *K. pneumoniae* ATCC 700603 strain was used as positive control in molecular tests.

Antimicrobial sensitivity

The susceptibility of the isolated strains to antimicrobials was evaluated according to the Kirby-Bauer Disk Diffusion Method (Bauer, 1996) and the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (PA, 2010). Bacterial suspensions were adjusted to 0.5 McFarland Standard and planted on Mueller Hinton Agar. Detection of Extended Spectrum β-Lactamase (ESBL) production was performed using the double disk synergy method (PA, 2010).

DNA Extraction

Boiling and freeze-thawing protocol, a simple, efficient, reproducible and inexpensive technique was used to extract DNA from each colony (Hasibuan et al., 2018). Isolated DNA contents were stored at -20°C until use.

Molecular typing

Following morphological and biochemical characterizations, identification of suspected colonies was further performed by *gyr*A gene PCR amplification specific for *K. pneumoniae* subsp., and *peh*X specific for *K. oxytoca*. Primers used in PCR analysis are presented in Table 1.

Table 1. Primers used in the study

Amplified Gene or Sequence	Oligonucleotide sequence (5'-3')	Product Length (bp)	References
gyrA[1]	F-CGCGTACTATACGCCATGAACGTA, R-ACCGTTGATCACTTCGGTCAGG	441	(Brisse & van Duijkeren, 2005)
K. pneumoniae[2]	F-ATTTGAAGAGGTTGCAAACGAT, R- TTCACTCTGAAGTTTTCTTGTGTTC	130	(Liu et al., 2008)
pehX[3]	F-ATACGGAGTATGCCTTTACGGTG, R-TAGCCTTTATCAAGCGGATACTGG	343	(Younis et al., 2017)
KPC	F- TCGCTAAACTCGAACAGG, R- TTACTGCCCGTTGACGCCCAATC	785	(Monteiro et al., 2009)
OXA	F- TGTTTTTGGTGGCATCGAT, R- GTAAMRATGCTTGGTTCGC	177	(Monteiro et al., 2012)
VIM	F-GATGGTGTTTGGTCGCATA R- CGAATGCGCAGCACCAG	382	(Poirel et al., 2007)

[1]*gyrA* = is for *Klebsiella* detection at genus level; [2]*K. pneumoniae* = Specific for *K. pneumonia* detection; [3]*pehX* = Specific for *K. oxytoca* detection.

PCR and Multiplex-PCR Protocols

To determine isolates at genus and species level molecularly, PCR was performed in 50 µL volume contained 5 µl target DNA, 10 pmol of each primer, 10 µl 5 × Master Mix (Solisbiodyne, Estonya), and 33,6 µl of ultra-pure distilled water. Thirty-five cycles with a profile of 95°C for 15 min (denaturation), 94°C for 30 sec (second denaturation), 58°C for 90 sec (annealing), and 72°C for 90 sec (extension) were run on a Techne PCR thermal cycler (BIORAD T100). Cycling was preceded by a final extension at 72°C for 10 min. Amplified PCR products were analyzed by electrophoresis in ethidium bromide-stained 1.5% agarose (w/v) gels and visualized with a UV-transilluminator.

Multiplex PCR Detection of Carbapenemase Genes

Primers designed to amplify the 3 genes encoding carbapenemases (*blaKPC, blaOXA and blaVIM*) were used (Ellington et al., 2007). The PCR mixture contained 5 µL DNA, 10 µL 5× Master Mix (Solisbiodyne, Estonia), 10 pmol of each primer, 34 µl ultrapure water was used.

RESULTS AND DISCUSSIONS

Of 1206 cows, 51 were clinically infected and bacteriologically positive. 20 of 51 clinical mastitis cases were culture-positive for *Klebsiella*. These results were confirmed molecularly and are shown in Figure 1.

CMT as a screening test indicates that 124/1206 (10.28%) cows and 180/489 (36.8%) quarters examined (except for 7 breast lobes - blind) were observed positive for subclinical mastitis. Out of 124 CMT-positive cows 18/124 (14.52%) cows and from 72 quarters 18/72 (25%) quarters were positive for *Klebsiella* isolation (Table 2).

Table 2. Prevalences of mastitis in the farms, Konya

%	Prevalence* in Farms					
Mastitis caused by	A	B	C	D	E	G
n	200	250	342	135	137	142
K. pneumoniae	1	3.2	2.04	1.48	5.83	0
K. oxytoca	0	1.6	0	0	0.73	0
Other *Klebsiella*	0	0	0.88	0	2.19	0
Total	1	4.8	2.92	1.48	8.75	0

The CMT produced statistically significant results on type of the farms (p > 0.05). Aerobic bacteria grow more in milk samples from manure littered bans. There is no difference between the numbers of coliforms from the farms using either robotic milking or classical milking systems (p > 0.05). This was also the case for the of aerobic bacteria numbers for the same farms (p > 0.05).

In farms that robotic milking systems was in use the negativity value for mastitis infection by *Klebsiella* was statistically quite low (p < 0.05). More *Klebsiella* spp. isolations occurred in the manure litter groups.

In farms equipped with milking systems, a correlation analysis was made between the some infectious/physiological data (intramammary subclinical and clinical mastitis status of dairy cows, lactation numbers of milkers, milking day after birth and milk yield) and isolations of *Klebsiella* spp. Between the isolation of *Klebsiella* spp. and day of milking a moderate negative correlation (r = -237) was observed with a P value of 0.016. Thus, as the milking day increases the *Klebsiella* spp. detection rate decreases.

Of the strains, 11 of the 16 ESBL positive isolates (64.28%) were found in farm B. No ESBL enzyme detection was seen in farms A and D. Ten of the strains with ESBL positivity were obtained from clinically mastitic cows. Molecular results regarding carbapenem resistance of isolates obtained from various sources are shown in Figure 2 and Table 3. The antimicrobial resistance profiles of the isolates, categorized by their sources of isolation, are presented in Table 4.

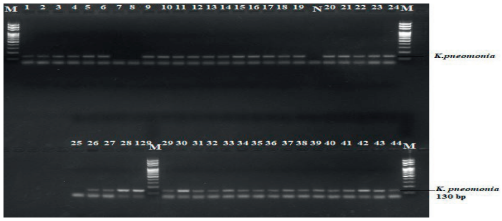

Figure 1. *K. pneumoniae* species-specific 16S-23S gene gel image of isolates, M: 100 bp DNA ladder

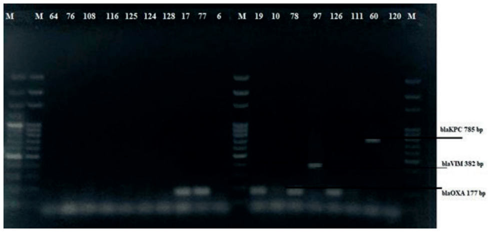

Figure 2. Gel image of carbapenem genes of isolates, M: 100 bp DNA ladder

Table 3. Distribution of carbapenem positive samples determined molecularly

Sources	*blaKPC*	*blaVIM*	*blaOXA*
Nasal (n = 45)	12*/17**	8*/17**	4*/17**
%	70.59	47.06	23.52
Rectal (n = 25)	5*/8**	5*/8**	2*/8**
%	62.5	62.5	25
Milk (n = 44)	1*/14**	4*/14**	10*/14**
%	7.14	28.57	71.43
Organ (n = 6)	1*/3**	1*/3**	1*/3**
%	33.33	33.33	33.33
Environmental (n = 8)	0*/2**	0*/2**	2*/2**
%	0	0	100
Total	19	18	19

*Test Result
**Total Number of Samples

Table 4. Percent resistances of the isolates to antimicrobials based on isolation sources

Antimicrobial	Milk	Nasal	Rectal	Environmental	Organ
AMC	29.5	44.4	48	16.7	75
C	36.4	13.3	8	16.7	87.5
PY	79.5	88.9	88	83.3	87.5
T	54.5	42.2	48	83.3	87.5
IPM	18.2	20.0	12	50.0	0
SXT	34.1	13.3	12	33.3	87.5
E	100	100	100	100	100
CN	13.6	8.9	16	0	87.5
AM	100	100	100	100	100
CTX	38.6	20.0	16	16.7	25
N	45.5	55.6	36	16.7	62.5
FLM	15.9	6.7	20	33.3	87.5
Colistin	22.7	31.1	12	33.3	50

AM10: Ampicillin (10 mg), PY100: Carbenicillin (100 mg), CTX30: Cephataxime (30 mg), C30: Chloramphenicol (30 mg), E15: Erythromycin (15 mg), CN10: Gentamicin (10 mg), N30 Neomycin (30 mg), T30:Oxytetracycline (30 mg), SXT25: Sulphamethoxazole/ Tripethoprim (1.25/23.75 mg), AMC30: Amoxicillin- Clavulanate (30 mg), IPM10: Imipenem (10 mg), FLM: Flumequin (30 mg)

Housing conditions, bedding material, milking systems, and udder hygiene are all critical environmental factors that directly affect udder health. The environment is sometimes itself the factor that allows some amount of pathogens to enter the udder tissue and thus increases the risk of infection (Srivastava & Kumaresan, 2015). *K. pneumoniae* is considered an opportunistic pathogen that poses a risk to humans and animals as evidenced by other studies so far (Fu et al., 2022; Mirzaie & Ranjbar, 2021). However, epidemiological studies of bovine mastitis caused by *Klebsiella* spp. are still insufficient in many part of the World (Fu et al., 2022). A complete history of bovine *Klebsiella* mastitis prevalence in Turkey is described elsewhere (Atalay, 2023). From 1979 on, occurrence has been reported between 0-17.9% depending on the city and year. In Konya, the detection of *Klebsiella* spp. as causative of bovine mastitis was first reported in 1992. No declining trend in the occurrences of *Klebsiella* mastitis in cattle can be observed in the Country anyway (Atalay, 2023)

All of the farms in this study were free from bovine Tuberculosis and Brucellosis, use automated milking systems and contained 100 lactating cows/each at least. One (Farm E) of the farms sampled that was bedded with manure and with free stall, open roof, shed-typed barn gives the highest prevalence of *Klebsiella* spp. mastitis ($p<0.05$). Percentages of mastitis (subclinical and clinical) cases in total according to farms A, B, C, D, E, and G were as follows; 15% (12.5%; 2.5%), 21.2% (14.8%; 6.4%), 9.06% (5.26%; 3.80%), 13.33% (8.14%; 5.18%), 16.05% (12.14%; 3.65%), and 14.78% (11.26%; 3.52%), respectively. However, Farm G was unique in the characteristics that no *Klebsiella* mastitis was detected at all although mastitis (either of type) is present (14.78%) in this farm with similar ratios to other farms. This might be because of that Farm G has more strict entry and exit allowances. On the other hand, by performing statistical analysis of the CMT results based on the litter type of the farms; differences were not statistically significant ($p > 0.05$).

In parallel with previous reports, as the day on lactation period increases, the detection rate of *Klebsiella* spp. decreases ($p < 0.05$). This supports the fact that coliforms causes mastitis in the early stages of bovine lactation since *Klebsiella* is one of the genera included in Coliform bacteriae (Constable et al., 2016). This early stage of vulnerability to *Klebsiella* spp. may be due to an increased susceptibility of the host or some other host specific reasons such as changed levels of lactoferrin and citrates (Burvenich et al., 2003). However, *K. pneumoniae* is proposed to be superior to most *E. coli* strains in bypassing the barriers posed by lactoferrin and infiltrating the mammary gland. On the other hand, similar to a *E. coli* infection, *K. pneumoniae* infection usually begins with subclinical mastitis at the end of the dry period, which can develop into a clinical form with the onset of lactation (Bradley & Green, 2000).

In Turkey, ESBL has been noted to be detected in one *K. oxytoca* isolate and four *E. coli* isolates obtained from mastitic cows (Babacan, 2022). At the present study, ESBL-producing strains were detected in 12.5% of all *Klebsiella* spp. isolates obtained from cattle. The corresponding figure for milk samples in total was 25%. The rates of ESBL enzyme in the *Klebsiella* obtained from lactating cows with clinical or subclinical mastitis were very close to each other (25.9%, 23.53%).

It was reported more than 10 years ago that coliform bacteria (*E. coli, Klebsiella* spp.) were 100% sensitive to carbapenems (Büyükcangaz et al., 2012). In the present study, 9 of 16 ESBL-positive isolates were resistant to Imipenem (IPM). IPM resistance was 17.9% when all of the isolates were taken into account. Two of the resistant strains obtained from the environment originated from the walls of animal shelters. These environmental isolates were from only one farm. The farm where we detected IPM resistance from the environmental samples was the farm that the highest rate of 55% IPM resistance and the highest rate of 69% ESBL enzyme occurred together. This suggests that it seems to pose a risk in terms of transmission to and between animals. Some of the primary protection practices, namely udder washing, drying, and teat dipping must be practiced in dairy farms. Testing cows by CMT is also crucial to detect early subclinically infected cows. A new introduction into the herd should not be allowed in any circumstances, either. Protection of udder health by the administration of proper vaccines is recommended as the first barrier to specific pathogens, too. Therefore, all these primary protection measures make the preventive approach in question indispensable to the dairy industry.

The development of antimicrobial resistance in veterinary medicine is driven by the wide utilization and misuse of antibiotics (Bedawy et al., 2024). The presence of the natural existence of β-lactamase in *K. pneumoniae* can explain the resistance to β-lactams and 3rd and 4th generation cephalosporins (Marr & Russo, 2019). Additionally, extended-spectrum β-lactamases (ESBLs) in this bacterial species is a well-known phenomenon as one of the plasmid-mediated enzymes (Bedawy et al., 2024; Koovapra et al., 2016). When extended-spectrum beta-lactamase activity was examined in *Klebsiella* strains, it was determined that bacteria with this enzyme spread in the environment and this feature could pass on to other bacteria quickly. This spread can occur between similar or different bacterial species (Caniça et al., 2015; DuPont & Steffen, 2017). It has been pointed out that animal foods play an important role in the spread of such bacteria among humans (Wu et al., 2013). In Farm B in this study, the antibiotics used must be resistant to the β-lactamase enzyme since ESBL had the highest rate of positivity with 69%. Therefore, antibiotics that would be used in combination with β-lactamase inhibitors such as sulbactam and clavulonic acid may increase their effectiveness. Detection of β-lactamase positivity before starting treatment is critical for identifying resistant isolates.

Carbapenems are recommended for high risk community acquired and nosocomial infections (Cole et al., 2022; Logan & Weinstein, 2017). Carbapenemase positive bacteria have been proposed to sometimes exhibit only a slight increase of MIC values for carbapenems. Genotypic rather than phenotypic methods to detect resistance to carbapenems is more convenient (Cornaglia et al., 2007). The present study shows that carbapenem resistance is evidenced genotypically in all of 6 dairy farms sampled and frequency was between 2-34%.

In the present study, the identification of isolates positive for carbapenem resistance genes from the same animal's either rectal or nasal samples along with a finding that a high rate of such resistance was seen in a particular farm together reveals that a potential risk of contamination between the cows is present.

Plasmid-mediated transfer of codes for enzymes may enable the transfer of resistance between bacterial populations. In our study, the carbapenem resistance gene was found in approximately one-third of the *Klebsiella* isolates from milk-originated samples (31.81%, n = 44). Although no animals in Konya are known to be treated with carbapenem-based antimicrobials, this study suggests that *K. pneumoniae* carrying genes for resistance to carbapenems and third-generation cephalosporins present in dairy cows can have critical potential for pathogenicity for humans. The fact that *K. pneumoniae* carries the same virulence genes in both animals and humans indicates a possible zoonotic potential of this microorganism. It may mean that the infection that can be mutually transmitted between animals and humans cannot be ignored.

The European Antimicrobial Resistance Surveillance Network (EARS-Net) performs surveillance of antimicrobial susceptibility of eight bacterial pathogens in humans including *K. pneumoniae*. To strengthen health surveillance throughout the whole of Europe, EARS-Net and the European Antimicrobial Resistance Surveillance Network in Veterinary Medicine (EARS-Vet) have been decided to collaborate and started to work together. As an outcome of this collaboration an alert on how carbapenem-resistant *K. pneumoniae* is transmitted between animals and humans was set (Mader et al., 2021). We recommend establishing a surveillance program covering both bovine and human isolates within the framework of *One Health*, in Turkey.

In this study, colistin resistance was determined as 22.7%, 31.1%, 33.3%, 12% in milk, nasal, environmental and rectal samples, respectively. This type of resistance was not found in nasal, rectal and milk samples from two farms (A and C). We believe that this has been interpreted as the colistin resistance may occur based on farm management practicals (those in which colistin was in use).

CONCLUSIONS

The detection of carbapenem resistance genes in both animal and environmental samples poses a significant risk to animal and public health, suggesting potential horizontal gene transfer. These findings support the implementation of comprehensive surveillance programs covering both animal and human health sectors, in line with the One Health approach, to effectively manage and reduce the spread of resistant pathogens.

To treat specific infections caused by multiresistant Gram-negative bacteria few drugs are recommendable. Carbapenems are of great importance and are used to treat these kinds of infections. Traditionally, those caused by extended-spectrum beta-lactamase (ESBL) producing bacteriae that are members of Order *Enterobacterales* and multi-drug-resistant organisms, such as *Pseudomonas aeruginosa* and *Acinetobacter* spp. are prescribed.

Carbapenem-resistant organisms (CRO) have recently become a significant threat to human health and healthcare systems. In human medicine, controlling CROs is seen as a priority issue, necessitating a multifaceted approach that includes aggressive infection control strategies, enhanced surveillance, and more effective antimicrobial stewardship measures.

The use of carbapenems in veterinary medicine should be out of choice since it is off-label. Thus it has been common to consider carbapenem as a reserved antibiotic only for cases with limited therapeutic alternatives. Carbapenem is not routinely prescribed for veterinary treatment purposes in Turkey.

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VALIDITY OF HEMOGLOBIN ESTIMATION METHODS FOR CHOLISTANI CATTLE BLOOD: A METHOD-COMPARISON STUDY

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Abstract

The present study was conducted with objective to evaluate the validity of three hemoglobin (Hb) estimation methods, including the cyanmethemoglobin method (HbC), Sahli's method (HbS), and veterinary hematology analyzer (HbA) in Cholistani cattle (n=100). Blood samples were collected aseptically from apparently healthy cattle. The results regarding the overall data and the data for age, and sex-wise groups revealed that HbA was significantly ($p \leq 0.05$) different from HbC, whereas HbS was non-significantly ($p \leq 0.05$) different from the HbC. The Bland and Altman chart between HbS and HbC, and between HbA and HbC showed significantly higher level of agreement between HbS and HbC with no proportional bias on the distribution of data around the mean difference line (Mean= 0.39, 95% CI= 0.21 to 0.57). Cronbach's alpha and intraclass correlation coefficient between HbS and HbC, and between HbA and HbC for single and average values, on similar grounds, were also higher between HbS and HbC being 0.819 and 0.900 as compared to the values of 0.793, and 0.884 between HbA and HbC. Sahli's method (a three-time average) for Hb estimation in cattle blood is comparable to the gold standard technique of the cyanmethemoglobin method, endorsing its use as a point-of-care testing device in remote areas.

Key words: *Cholistani cattle, hemoglobin, point-of-care testing.*

INTRODUCTION

Hemoglobin (Hb) is a tetrameric structural and functional unit formed by the asymmetric pairing of two polypeptide chains, the alpha and beta globulins. Within the erythrocytes, it generates carbamino compounds with carbon dioxide and buffers hydrogen ions, facilitating carbon dioxide transport in blood (Brundha & Priyadharshini, 2019). Priorly, extensive reviews have emanated globally which have reviewed the merits and demerits of various Hb estimation methods mainly the Tallquist method, Copper sulphate method, Sahli's method, Lovibond comparator, cyanmethemoglobin method, Hb color scale, and HemoCue (Srivastava et al., 2014). Their precision, accuracy, sensitivity, specificity and repeatability for human blood has also been reported (Barduagni et al., 2003; Adam et al., 2012; Agnihotri et al., 2015). In comparison to these methods, lately the 3-part and 5-part automated veterinary hematology analyzers are frequently being used to determine Hb levels in human and veterinary hematology. These machines, though highly accurate and reliable, are yet costly, tedious and need trained personnel. Furthermore, the transfer of blood samples to the laboratory may delay treatment, resulting in disease aggravation (Adam et al., 2012). The use of such analyzers has resultnalty quite limited in resource-poor countries (including Pakistan) owing to aforementioned limitations.

The World Health Organization conducted research on Hb determination using the Hb color scale in which a color of the drop of blood was compared to specified red shades (Darshana & Uluwaduge, 2014). The scale comprises of a little card with six different red colors representing Hb levels of 4, 6, 8, 10, 12, and 14 g/dL. HemoCue portable photometer is another way for determining Hb. It comprises of disposable microcuvettes holding dry reagent and a single-purpose photometer. The precision of HemoCue for assessing Hb concentration in venous or capillary blood samples was inferior and was not equivalent to that of an automated hematology analyzer (Kapoor et al., 2002).

The cyanmethemoglobin technique is widely regarded as the gold standard for Hb estimation. It can measure all forms of Hb except sulfhemoglobin. However, there are several drawbacks of using this method, as it may be toxic due to presence of cyanide in its reagent, presence of turbidity, and it requires skilled technician and presence of unique equipment (Kapoor et al., 2002; Srivastava et al., 2014).

Sahli's approach is doable by hand. It is less expensive, less time consuming, more convenient, and simpler to carry out. As a result, it is a superior choice for on-field investigations rightly coming under the definition of 'point-of-care testing device' (POCT) (Singh et al., 2015) However, visual mistake is likely when matching the brown hue of the comparator box in this procedure, and all types of hemoglobin cannot be measured (Balasubramaniam & Malathi, 1992).

Cholistani cattle are phenotypically a big, with a stumpy body, having short horns, long ears, and substantial dewlap both in males and females. Males possess a prominent hump. The body of this breed of cattle is speckled with red, black, or brown dots, and its tail features a black switch. The genetically superior Cholistani cows may produce 15 to 18L of milk each day (Farooq et al., 2010). This breed gained its fame since 2010 and extensive research work on its reproductive and productive attributes has since been reported from Pakistan (Tausif, 2008; Ali et al., 2009; Shahzad et al., 2010) Lately, our laboratory has published results regarding the reference intervals for various hematochemical profile of apparently healthy Cholistani cattle being reared under nomadic pastoralism in the Cholistan desert of Pakistan (Saeed et al., 2022). Furthermore, our laboratory has also initiated work on validation of various hematological attributes and deducing pen-side hematological formulae for various Cholistani livestock (Ahmad et al., 2022a; Ahmad et al., 2022b; Farooq et al., 2023). However, no work has yet been reported on assessing the diagnostic efficacy of various Hb estimation methods for this breed of cattle which may be used as point-of-care testing (POCT). The present study is therefore being devised with an objective to assess the validity of various Hb estimation methods (Sahli's method, cyanmethemoglobin method, automated veterinary hematology analyzer) for blood of Cholistani cattle being reared under pastoralism in Cholistan desert, Pakistan.

MATERIALS AND METHODS

The research work was conducted at the Cholistan desert (for blood sampling) and post-graduate laboratory of the Department of Physiology, Islamia University of Bahawalpur (for analyses). The cyanmethemoglobin technique is regarded as the gold standard for Hb detection. Pakistan throughout the time of June 2022 to May 2023. This desert has an area of 26,000 km^2 and is located in latitudes 27°42′ and 29°45′ North, longitudes 69°52′ and 75°24′ East, and at a height of roughly 112m above sea level (Farooq et al., 2017). It comes under the domain of semi-arid tropical climate with average temperature of 28.33°C. Month of June is considered as the warmest month with a temperature soaring beyond 45°C (Farooq et al., 2010). Cholistan has an annual rainfall of up to 180mm. November through January are coldest of the months having an average temperature of 13°C. Cholistani cattle (n = 100) being reared by the desert nomads of Cholistan were incorporated in this study. Detailed interviews were conducted with these nomads and clinical assessment of the animals, the overall health of the animals was assessed, and only apparently healthy animals were included in the research. According to the anamnesis provided by pastoralist herders, animals deemed to be listless, depressed, off-feed, and separated from the herd were excluded from the research. Demographic information was gathered through focal group discussion from the livestock herders.

As directed by protocol, a disposable syringe was used to take blood samples from the cattle's jugular vein. After being carefully inverted and placed in an icebox, the blood samples were moved into purple-topped EDTA-containing vacutainers (Becton Dickinson, USA) and examined for hematological analysis within eight hours. Three approaches were suggested in this study to estimate the Hb levels in the blood of Cholistani cattle; as given below:

a) Sahli's method: The Sahli's approach includes converting Hb to acid hematin and visually comparing the resultant color to that of conventional colored glass. The value of Hb

(HbS) was read straight from the graduated Hb tube (Balasubramaniam & Malathi, 1992). Three trained personnel took this reading separately in order to eliminate interpersonal errors and the reading was taken in broad daylight. Mean of these readings was taken into account.

b) Cyanmethemoglobin method: For the cyanmethemoglobin method, about 5mL of Drabkin's solution (SDL Company, Pakistan) was taken by adding 20 µL blood into it. It was mixed thoroughly and incubated for 5 minutes. After that, the reading was taken through spectrophotometer (Irmeco U2020, Germany) set at 540 nm wavelength (Sari et al., 2001) and Hb was deduced through standard curve (HbC). This HbC was considered as gold standard method for Hb determination.

c) Automated veterinary hematology analyzer: Blood was well mixed on a Roller Mixer (MixR-40, Daihan Scientific, Korea) and then subjected to an off-hand validated automated veterinary hematology analyzer (Rayto RT-7600, China) to determine the hemoglobin concentration (HbA).

Data analysis was done using the Statistical Package for Social Science (SPSS for Windows version 12, SPSS Inc., Chicago, IL, USA). Using the Shapiro-Wilk test, the normality of the data was examined. Using the provided formulas, the means (± SE) and 95% CI for the three Hb values (HbS, HbC, and HbA) obtained in this study were calculated. ANOVA with Duncan's as a post-hoc test was suggested in order to determine the difference between the three Hb values for the total data as well as for the different study groups, namely age-wise (young, n = 45 and adults, n = 55) and sex-wise (males, n = 68 and females, n = 32). The degree of correlation between the three Hb levels was calculated using Pearson's correlation coefficient. Scatterplots were drawn and linear regression was carried out between these three Hb values and accordingly, regression prediction equations were computed. Considering cyanmethemoglobin as gold standard method of Hb estimation, level of agreement between HbS and HbC, and between HbA and HbC was assessed through Bland Altman agreement analysis (Bland and Altman, 1999). Likewise, as tests of agreement between HbS and HbC and between HbA and HbC, Cronbach alpha and intraclass correlation were also deduced from the whole data (Gerke, 2020; Shieh, 2020).

RESULTS AND DISCUSSIONS

The mean (± SE) values and 95% CI for the three Hb values attained in this study (HbS, HbC and HbA) in Cholistani cattle (n = 100) are given in Table 1.

Similarly, results for overall and group-wise (based on age and sex) data revealed that HbA was significantly ($p \leq 0.05$) different from the HbC, whereas HbS was non-significantly ($p \leq 0.05$) different from the HbC.

Table 2 displays the regression analysis results and the corresponding regression prediction equations for each age and sex group under consideration. A significant positive correlation coefficient ($p < 0.01$) was observed between HbA and HbC as well as between HbS and HbC. The greatest association between HbS and HbC, however, was found in adult stock (r = 0.866; adjusted r-square = 0.743).

Table 1. Mean (± SE) values and confidence intervals for hemoglobin determined through Sahli's, cyanmethemoglobin method and autoanalyzer

Groups		HbS (g/L)		HbC (g/L)		HbA (g/L)	
		x ± SE	CI	x ± SE	CI	x ± SE	CI
Sex	Females (*n* = 32)	100.0 ± 1.9a	96.3-103.8	102.1 ± 2.0a	98.0-106.2	105.0 ± 2.1b	100.7-109.4
	Males (*n* = 68)	98.0 ± 1.8a	94.3-101.7	105.9 ± 1.8a	102.1-109.6	99.6 ± 1.8b	95.8-103.4
Age	Young (*n* = 45)	96.9 ± 1.5a	93.9-99.9	103.0 ± 1.6a	99.7-106.2	99.7 ± 1.5b	96.6-102.7
	Adults (*n* = 55)	101.5 ± 2.2a	97.0-106.0	103.5 ± 2.4a	98.7-108.4	106.3 ± 2.6b	101.1-111.4
Overall (*n* = 100)		99.4 ± 1.4a	96.6-102.2	103.3 ± 1.5a	100.3-106.3	103.3 ± 1.6b	100.1-106.5

[a,b]Different letters within rows are different at $p \leq 0.05$ for the three hemoglobin values. HbS= Hemoglobin determined through Sahli's method; HbC= Hemoglobin determined through cyanmethemoglobin method, HbA= Hemoglobin determined through veterinary hematology analyzer; CI= Confidence interval.

Table 2. Linear regression between hemoglobin determined through Sahli's method, cyanmethemoglobin method and veterinary hematology analyzer in Cholistani cattle (n = 100)

Groups		HbS versus HbC	R	Adjusted r Square	HbA versus HbC	r	Adjusted r Square
Sex	Females (n = 32)	y=0.791(HbC)+1.9	0.860**	0.736	y=0.886(HbC)+1.4	0.848**	0.714
	Males (n = 68)	y=0.702(HbC)+2.3	0.708**	0.485	y=0.726(HbC)+2.2	0.711**	0.489
Age	Young (n = 45)	y=0.637(HbC)+3.1	0.707**	0.488	y=0.555(HbC)+4.2	0.597**	0.341
	Adults (n = 55)	y=0.795(HbC)+1.9	0.866**	0.744	y=0.910(HbC)+1.2	0.865**	0.743
Overall (n = 100)		y=0.761(HbC)+2.0	0.821**	0.671	y=0.832(HbC)+1.73	0.794**	0.626

**Significant correlation at $p \leq 0.01$. HbS= Hemoglobin determined through Sahli's method; HbC= Hemoglobin determined through cyanmethemoglobin method, HbA= Hemoglobin determined through veterinary hematology analyzer.

The Bland and Altman chart between HbA and HbC (Figures 1 and 2, respectively) and between HbS and HbC (Figure 1) indicated a better level of agreement with respect to the level of agreement.

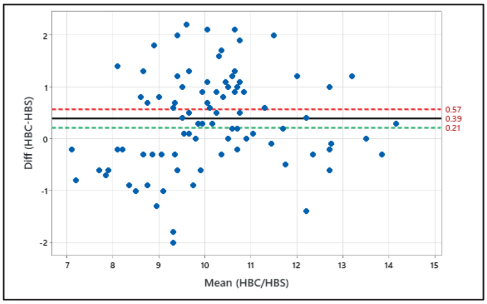

Figure 1: Scatterplot of Bland and Altman Test between Difference of Hemoglobin Determined through Cyanmethemoglobin (HbC) and through Sahli's Method (HbS) (HbC-HbS) and Average of Both Hemoglobins (HbC+HbS/2). Black line indicates mean difference (0.39) whereas the upper red and lower green lines indicate upper (0.57) and lower (0.21) values for 95% CI, respectively

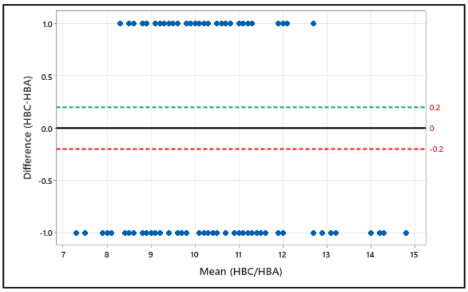

Figure 2: Scatterplot of Bland and Altman Test between Difference of Hemoglobin Determined through Cyanmethemoglobin (HbC) and through Veterinary Automated Hematology Analyzer (HbA) (HbC-HbA) and Average of Both Hemoglobins (HbC+HbA/2). Black line indicates mean difference (0.0) whereas the upper green and lower red lines indicate upper (0.2) and lower (-0.2) values for 95% CI, respectively

Additionally, there was no discernible proportional bias in the data distribution along the mean difference line between HbS and HbC (Mean = 0.39, 95% CI = 0.21 to 0.57).

Table 3 presents the findings for the intraclass correlation coefficient and Cronbach alpha between HbA and HbC and between HbS and HbC. The average and single measure values between HbA and HbC were 0.793 and 0.884, respectively, while the values between HbS and HbC were higher at 0.819 and 0.900.

Table 3. Cronbach alpha and intraclass correlation between hemoglobin determined through Sahli's method, cyanmethemoglobin method and autoanalyzer

HbS and HbC			
Intraclass Correlation		95% CI	Cronbach Alpha
Single Measure	0.819	0.74-0.87	0.900
Average Measures	0.900	0.85-0.93	
HbA and HbC			
Single Measure	0.793	0.70-0.85	0.884
Average Measures	0.884	0.82-0.92	

HbS= Hemoglobin determined through Sahli's method; HbC= Hemoglobin determined through cyanmethemoglobin method, HbA= Hemoglobin determined through veterinary hematology analyzer

Blood analysis is one of the vital and precise tools being used widely in medical practice. Therefore, globally, clinical hematology both for human and veterinary medical sciences has gathered considerable footing. And it has laid precise foundations of diagnosis/prognosis of blood-borne disorders. The development of 3-part and 5-part automated veterinary hematology analyzers has replaced the manual hematology methods such as measuring RBC and WBC using hemocytometers, packing cell volume through microcentrifugation (Organization, 2000), differential leukocyte count through stained blood smears (Hu et al., 1993), and measuring Hb levels through cyanmethemoglobin (Kapoor et al., 2002; Srivastava et al., 2014). However, the expensiveness, periodic maintenance, need for trained personnel, continued validation, and expensive chemical reagents for these analyzers deem these analyzers unfit to be utilized as a POCT devices. Being portable, easy to use, and provision of quick results, the POCTs are in high demand especially in developing/under-developed parts of the world which either have restricted or limited excess to standard laboratory analyses (Chevalier et al., 2003; Abuelo & Alves-Nores, 2016) The present study assessed the validity and diagnostic accuracy of three Hb estimation methods (Sahli's method, cyanmethemoglobin method, automated veterinary hematology analyzer) for Cholistani cattle blood. The Sahli's hemoglobinometer, which is a POCT device for Hb estimation in humans, gave results for Hb which were similar to those attained through the automated veterinary hematology analyzer in the present study. This endorses its vitality as a POCT device for Hb estimation in cattle blood. There is almost no work conducted on the study of diagnostic accuracy and efficacy of Sahli's hemoglobinometer for cattle blood, hence, as per need, the results of the present study have been compared with prior studies conducted on human blood.

The results for overall data as well as for age and sex-wise groups in the present study showed that the Hb values for Cholistani cattle blood attained through automated veterinary hematology analyzer (HbA) were significantly different from those attained through cyanmethemoglobin method (HbC) and through Sahli's hemoglobinometer (HbS). However, the values for HbS and HbC were non-significantly different within each other, indicating a substantial diagnostic accuracy and efficacy of Sahli's method for this breed of cattle. Our results are not in line with most of the research work conducted on humans (Sari et al., 2001) and animals (DeNicola, 2011) which have shown better efficacy and diagnostic accuracy of auto-analyzers as compared to Sahli's method. Visual impairment of the observer, less light, and fading out of the comparator-color-block of the Sahli's hemoglobinometer are few of the drawbacks which decrease its efficacy for Hb estimation (Critchley & Bates, 2005). Sahli's method is a subjective test based on visual comparison and has a lower sensitivity, specificity, positive predictive value and negative predictive value as compared to other Hb estimation methods such as cyanmethemoglobin method and auto-analyzers (Barduagni et al., 2003; Brundha & Priyadharshini, 2019). A digital hemoglobinometer, in a study, was found to have lower sensitivity and specificity of 89.4% and 63.6% for pregnant women in India, respectively as compared to 98.7% and 90.2% for autoanalyzer (Toppo et al., 2019) Similarly, while comparing Sahli's method with cyanmethemoglobin method for human adults, a lower sensitivity of 86.2% has been reported as compared to 96.5% for copper sulphate method (Agnihotri et al., 2015) Apart from all this, Sahli's method is still being used as a POCT device for clinical and research purposes, especially in resource-poor settings such as Asian countries, both for human (Sari et al., 2001; Wasnik et al., 2014) and veterinary (Pathan et al., 2011; Ibrhim, 2014; Osman et al., 2017) medical sciences.

An interesting study conducted in India compared the efficacy of Sahli's method (two-time and three-time average) and autoanalyzer for Hb determination in human blood. It was reported that the three-time average of Sahli's method was statistically not different from that attained through autoanalyzer (Brundha & Priyadharshini, 2019). These results coincide with the results of the present study. In our study, average reading from three trained personnel was taken for Hb using Sahli's method which is not different from the gold standard technique of cyanmethemoglobin.

Regarding the studies on the efficacy of Sahli's method in livestock blood, the only study conducted in Iraq has compared Sahli's method against autoanalyzer for bovine, caprine and sheep blood (Ibrhim, 2014). This study has also reported that the values attained through Sahli's method are lower and statistically different than those attained through auto-analyzers for all three studied species. Another study has compared the efficacy of Mission Plus (MP) human device for Hb estimation with gold standard technique for healthy cattle blood using Passing Bablok regression analyses and Bland

Altman chart. They have reported that the human MP device is equally effective for Hb estimation as the gold standard technique for cattle blood (Heller et al., 2021).

In the present study, gold standard method of Hb estimation *i.e.* cyanmethemoglobin (HbC) was compared both with the HbS and HbA. Three main statistical tests *viz*. Bland and Altman, Cronbach alpha and Intraclass coefficient were implied for assessing the level of agreement within these three Hb values. No proportional bias on the distribution of data around the mean difference line was noticed between HbS and HbC, and there was a strong level of agreement between HbS and HbC, as compared to that between HbA and HbC. Hematology analyzers, these days, are extensively being used both by human and veterinary medical practitioners and researchers. These machines are highly sensitive machines and are validated by the manufacturers as per the (inter)national standards to be sent out into the market. However, their performance has been marred by their need of periodic validation and continued quality control measures. The difference in Hb values attained through veterinary hematology analyzer and through gold standard method in the present study may be indicative of the fact that the machines need periodic validation through manual hematological methods as reported earlier (DeNicola, 2011; Vis and Huisman, 2016; Kratz et al., 2019).

CONCLUSIONS

In a nutshell, the present study reveals that the value of Hb for cattle blood attained through Sahli's method is comparable to that attained through gold standard technique of cyanmethemoglobin method. This endorses the on-field use of this POCT device for Hb estimation in cattle being reared in far-flung areas for quicker, cheaper and reliable results. However, we recommend three-time average of the values taken through this device for better results. It is further recommended that other POCT devices for Hb estimation being used in human practice such as HemoCue, MissionPlus, and Hb color charts/scales, may also be validated for livestock blood.

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HEALTHCARE MANAGEMENT USING AMAZON WEB SERVICES

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Abstract

Healthcare and life sciences represent a significant role in people's lives, these two aspects governing their well-being and functioning. Redefining patient care methods and treatment solutions, working on thorough genomics studies and balancing healthcare costs have become an upfront priority for any healthcare and life science organization. A cloud infrastructure composed of applications, servers, networks, and data storage represents an effective solution to both business and technical goals, but also a modernization tool. For this study, we created a web-based program called HealthCloud, which facilitates medical data interoperability, provides automation services, and uses machine learning for predictions and insights related to the possibility of developing a cardiovascular disease, based on patient input regarding food and nutrition. Our results include a user-friendly interface, easy access to patient and doctor data, and a high accuracy of disease risk prediction with a machine learning model built in Amazon SageMaker. The proposed prototype proved its potential through the attained outcomes and presents an appropriate approach of healthcare management, which merges with cloud technology for the enhancement of care delivery.

***Key words**: Computer Applications, Digital Healthcare, Machine Learning, Supervised Learning, Unsupervised Learning.*

INTRODUCTION

The need for a digital transition in the healthcare management system has proved to be fast-forward and was mainly generated by the COVID outbreak (Hamilton, 2023). Now, the goal of health industry executives is to produce durable, cutting-edge medical systems, such that the challenges of today are surmounted with more resilience.

Not for a long time now, there have been some repeatedly occurring patterns that demonstrated the need for more creative solutions and tech related innovations (Pavaloiu et al., 2015).

Chronic Illnesses. According to the National Center for Chronic Disease Prevention and Health Promotion (NCCDPHP), a chronic disease is commonly defined as a medical condition that lasts 1 year or more and needs constant medical care (Centers for Disease Control and Prevention, 2002).

Death, disability along with highly expensive healthcare costs per year are consequences produced by major chronic diseases, such as diabetes, cancer, and heart disease (Culberson et al., 2023). This represents a challenge for the healthcare management systems, which are burdened with the increase of chronic diseases among an aging population: four in ten adults suffer from 2 or more chronic illnesses (Hajat & Stein, 2018). Multiple Chronic Conditions (MCC) is when a person lives with several chronic conditions and requires a complex healthcare management system due to its posing difficulties on someone's life (Caldeira et al., 2021). Chronic illnesses can also include mental health related issues, such as chronic depression, anxiety, bipolar disorder, memory problems and so on (Manger, 2019).

Physical and emotional illnesses can have a significantly worse outcome on someone's quality of life, making them unable to manage day to day activities, such as exercising, doing house chores, or even working. Large amounts of medications are required to reduce the pain and symptoms of the illness and keep it under control (Nijs et al., 2021). However, side effects occur, and it becomes more exhausting to manage such a disease that cannot be cured. This leads to depression and a never-ending cycle, which burdens the patient and the healthcare providers with ineffective medications and, respectively, exponentially growing expenses.

Telemedicine and remote patient monitoring (RPM). This challenge emerged at its peak during COVID-19 and highlighted the importance of several technologies such as Internet of Things (IoT), machine learning (ML), virtual reality (VR), and Big Data (Brahmbhatt et al., 2022). The most affected area following the pandemic is the healthcare industry and was forced to quickly adopt the expanded use of digital technologies (Darbandi et al., 2022). Subsequently, the usage of tele-medicine brought upon the healthcare sector additional issues: in underdeveloped locations, healthcare providers and patients experience hardship since the limited resources restrict them from using these technologies to their fullest potential (Ftouni et al., 2022). The integration of telehealth and remote patient monitoring into healthcare-related workflows and operations may pose difficulties: clinical staff might need additional training, there would be a disruption in the existing workflows, and healthcare leaders must document these new technologies and have the knowledge to modify clinical procedures accordingly (Pugmire et al., 2023).

High-level digitalization depends also on the patient's willingness to engage and adapt, particularly in the case of elderly patients, being a prevalent preference for an in-person encounter with healthcare professionals. Their understanding of digital literacy is limited; hence, the start or continuation of their virtual care treatment would be impeded.

Machine Learning and AI. Artificial Intelligence brings with it numerous benefits in healthcare, such as disease prediction, consistent routine check-ups, managing appointments with virtual healthcare assistants, and drug discoveries (Haleem et al., 2022). However, developing and maintaining such practices may call for expensive investments, advanced technology infrastructure and skilled labor (Gangwar & Reddy, 2023). Financial investments for the application of the AI field in healthcare include the acquisition of additional specialized software and tools for production and deployment, high-performance computing, and other expensive resources. Additionally, obtaining AI solutions implies hiring data science and machine learning experts who can implement models and algorithms to solve several use cases.

The most fragile companies are the small businesses, which have less resources at their disposal, being prone to collapse while trying to acquire and maintain AI capabilities.

Another drawback lies in the lack of accuracy that could be generated due to the small amount of data regarding certain illnesses, demographics, or environmental variables (Coccia, 2023). This could result in the wrong interpretation of a diagnosis and the incorrect administration of prescription medicine. In this case, human surveillance is needed to confront the problem of misdiagnosis, which is especially prevalent in a particular demographic area (Mackenzie et al., 2022).

Cost-related obstacles. A constantly growing structure of the healthcare system requires a considerable number of resources and costs: infrastructure needs to be maintained and the healthcare systems become more complex with the user expectations and demands, as the populations grow and age (Waitzberg et al., 2022). Additionally, the regular monitoring of patients with diseases such as chronic illnesses implies that healthcare organizations get hold of more advanced technologies and interventions, which are of higher cost.

Greater expenses do not necessarily mean a great outcome: it has been confirmed that the U.S., as opposed to other countries, spends more on healthcare, with 30% of the expenditures being considered waste (Sullivan et al., 2023). Most of the waste emerges from high amounts of expenses and can be a result from the following difficulties: inability to provide proper treatment, poor care organization, overtreatment or care of low value, failure in pricing, fraud, abuse, and administrative complexities (Tushar et al., 2023).

A Cloud computing solution is an effective way to reduce waste and costs and to improve the patient's experience within a clinic: siloed data systems are no longer wanted by healthcare companies, since the cloud can offer the ideal balance between efficiency and costs (Barbandi et al., 2022).

Security and Integrity of Health Information Systems. One of the most delicate factors in healthcare is the data and its security, which is threatened with the expansion of modern technologies, making the healthcare sector the most targeted area by cyberattacks (Usmani et al., 2023).

There are several methods in which hackers could harm the healthcare system and its patients: firstly, the dark web is a gateway to anyone who has the intention of breaking into a system, even with limited abilities (Chauhan et al., 2022). Blackmail, stealing and selling information or the total compromise of the normal operation of a health information system are major threats in terms of healthcare hacking. Data must be safeguarded internally and externally, due to its frequent exchange between multiple healthcare establishments and providers, which increases its vulnerability to digital attacks.

Networks where medical data is shared in this manner attract more data thieves, who can access the patient records and steal information such as billing details. It is imperative that one pays more attention to the protection of electronic health records (EHRs), which are more challenging to secure. In the Materials and Methods section, is discussed the hosting with AWS Amplify and the advantages of cloud hosting. There are also explored the application of machine learning in healthcare and its role in predicting cardiovascular disease risk. Amazon SageMaker and its tools, such as Principal Component Analysis (PCA) and the XGBoost Classifier, are introduced as part of the current proposed software solution. There is highlighted the integration of machine learning into web applications and the need for more detailed accuracy assessment based on patient data. In the last section are mentioned the potential future improvements, including automated image analysis, virtual assistants, and a patient-focused mobile application.

MATERIALS AND METHODS

A. *Traditional Web Hosting versus Cloud Hosting*

Web hosting services offer the possibility of a business launching its product into the market, by establishing its visibility with online presence. There are two practical solutions for an application to be available on the internet and these are the traditional web hosting or hosting in the cloud (Zala et al., 2022). The classic hosting environment is divided into two categories: dedicated and shared. By using a dedicated hosting approach, the client receives a fixed number of resources over which he has full control, on one or more servers.

Shared hosting is more common among small and medium-sized businesses which will be given a set of resources shared with some other websites, on only one server. In the present moment, there are plenty of small and medium companies with limited budgets that are looking for a new and effective way of hosting their website according to their business requirements (Chidukwani et al., 2022). The innovative approach is Cloud hosting, which is also used in the current software solution, which is proposed, having several advantages over the traditional hosting (Ali et al., 2022), as shown in Table 1.

Table 1. Comparison between Traditional and Cloud Hosting

Metric	Cloud Hosting	Traditional Hosting
Scalability	“Pay as you go” for usage	Almost no scalability, hardware dependent
Elasticity	Elastic and resilient due to redundancy	Limited, no elasticity
Performance	Not affected by problems with one application	A single point of failure
Cost	Server operation and maintenance among several parties	Costly as one must buy necessary equipment
Deployment time	Less time, it does not rely on hardware	Extended time for setting up servers
Internet connection	It is needed as fast and reliable	Not needed

B. *AWS Amplify*

For the proposed project, AWS Amplify was chosen as a hosting environment (dedicated to multi front-end web and mobile apps), to deploy the web application in the cloud (Amazon, 2023). Additionally, as a version control system, GitHub was selected.

The steps which were respected were to navigate to the management console and choose the AWS Amplify service, followed by the connection of the source code from a GitHub repository, in this case the “master” branch is chosen. Afterwards, AWS Amplify was enabled to deploy all files of the project's root folder, automatically.

In Figure 1, the three steps of the previous mentioned process were completed successfully.

This type of deployment implied public Cloud as the category of Cloud for the current project idea since the application runs on the public infrastructure of AWS. Moreover, there are some benefits brought to this type of hosting, namely easy deployment, as the process is simplified by the automation of services configuration and the setup of continuous deployment pipelines.

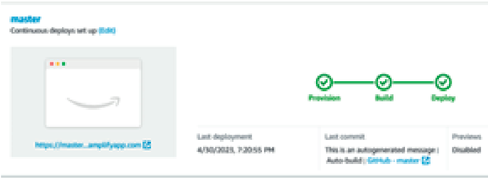

Figure 1. AWS Amplify Deployment
Source: own source

MACHINE LEARNING IN AWS

C. *Overview of ML in Healthcare*

The expansion of the healthcare industry depends recently on emerging technology approaches, such as artificial intelligence and machine learning, which allow the development of the field through fast diagnosis and a higher level of accuracy. It is worth mentioning that in the proposed software solution, both structured and unstructured data sets have been used to gain ML insights.

D. *Healthcare Dataset*

Before a diagnosis is made official, the doctor must make a thorough examination and analysis of the patient's mental or physical state. This assessment is done with the help of some medical data of the patient, which helps the healthcare provider to move in a particular path towards a certain diagnosis. To predict the presence or absence of risk for cardiovascular disease, a healthcare dataset from the National Institute of Aging (National Institute of Aging, 2023) has been used. This dataset encompasses an array of features crucial for gauging cardiovascular health.

The inclusion of objective features, subjective information, and examination results forms a multidimensional framework that aids in steering the diagnostic process in a targeted direction. It is important to note that this dataset does not operate in isolation; rather, it integrates various facets of the patient's health to provide a comprehensive overview.

From Table 2, one may observe that the input features are of three types, namely objective features represent factual data (age, height, weight, gender); subjective features which are provided by the patient (smoking, alcohol intake, physical activity); examination features which are discovered during the medical appointment (blood pressure, glucose, cholesterol).

Table 2. Features of used health dataset

Feature	Measurement
Age	int (days)
Height	int (cm)
Systolic blood pressure	int
Diastolic blood pressure	int
Weight	float (kg)
Gender	categorical code
Smoker	binary
Alcohol intake	binary
Physical activity	binary
Glucose	1: normal, 2: above normal, 3: well above normal
Cholesterol	1: normal, 2: above normal, 3: well above normal

Blood pressure (BP) is measured using two numbers: systolic and diastolic BP, where the first one must be less than 120 and the second one must be less than 80. Otherwise, in case of higher values, a cardiovascular disease might be present (Justin et al., 2022). Moreover, increased glucose and cholesterol levels also represent a risk factor in diabetes or a heart problem (Hariharan et al., 2022).

Besides the input features enumerated in the previous table, there is also the target variable, represented in binary, which marks the presence or absence of a cardiovascular disease. All the mentioned values were recorded during medical examinations.

The link between the input features and food and nutrition aspects of every person lies in the impact of dietary habits on objective and examination aspects associated with cardio-vascular health.

The systolic and diastolic BP are indicators of cardiovascular health that show the importance of maintaining a healthy blood pressure. Dietary factors such as high salt intake can contribute to elevated blood pressure (Robinson et al., 2019). Therefore, advising patients on a low-sodium diet can be crucial in managing blood pressure levels within the recommended range (O'Donnell et al., 2020).

Increased glucose and cholesterol levels are highlighted as risk factors for diabetes or heart problems (Eckel et al., 2021). Nutrition plays a significant role in managing these levels. A diet rich in fiber, low in saturated fats, and with controlled sugar intake can positively influence glucose and cholesterol levels (Samuel et al., 2023). Including foods like whole grains, fruits, vegetables, and lean proteins can contribute to

better overall cardiovascular health (Capurso, 2021).
Dietary needs vary based on age, gender, and body weight. Nutrition plays a vital role in supporting growth, maintaining a healthy weight, and addressing specific nutritional requirements based on gender (Wohlgemuth et al., 2021). Smoking and excessive alcohol intake are lifestyle factors that can negatively impact cardiovascular health (Kotseva et al., 2019). Proper nutrition, combined with lifestyle modifications such as regular physical activity, can contribute to reducing the risk of cardiovascular diseases (Lacombe et al., 2019).
Nutrition is a key factor in preventing and managing cardiovascular diseases. A diet rich in antioxidants, omega-3 fatty acids, and other heart-healthy nutrients can be recommended for individuals to reduce the risk of developing cardiovascular diseases (Szczepańska et al., 2022).

E. *Amazon SageMaker and ML Algorithms*

Amazon SageMaker is a cloud service belonging to AWS which makes simpler the process of building, training, and deploying ML models (Dubey & Dubey, 2022). It has the capability to process large amounts of structured and unstructured data and to significantly reduce the training time with a developed infrastructure.
The AWS ML Stack consists of AI, ML Services and ML Frameworks and Infrastructures. The ML Services include SageMaker Studio IDE, which enables the performance of all needed steps of the machine learning model (Ratan, 2022).
The disease prediction model of the current software solution was developed in a Jupyter notebook instance of SageMaker Studio.

F. *Principal Component Analysis Algorithm*

Principal Component Analysis (PCA) is an unsupervised machine learning algorithm that has as its goal to perform dimensionality reduction, where new features will be called components and will extract the most important information from the dataset (Ratra et al., 2022). This type of algorithm solves challenges in machine learning, such as overfitting, decreased accuracy, or increased computation time, since a high number of features in a dataset would result in an exponential growth in the data needed to obtain a meaningful result.
Regarding the proposed project, SageMaker PCA was used, which is an implementation of the traditional PCA, provided by AWS and has several additional features. Firstly, scalability, since the algorithm handles large datasets as well, as it functions in two modes: regular (for a smaller number of datasets and features) and randomized (for datasets with an increased number of features and observations).
Secondly, due to the interaction with other AWS Services, such as uploading training data to a S3 bucket. Lastly, hyperparameter optimization, by running many training jobs to determine the best model version. SageMaker PCA provides a list of hyperparameters for the training, such as *feature_dim*, which is the input dimension, *num_components* represents the number of principal components and *algorithm_mode* is the mode for calculating the components represented in Figure 2.

```
# reduce 11 features to 6 components
pca.set_hyperparameters(feature_dim=11,
                        num_components=6,
                        subtract_mean=False,
                        algorithm_mode='regular',
                        mini_batch_size=100)
```

Figure 2. PCA Hyperparameters
Source: own source

G. *eXtreme Gradient Boosting Classifier*

eXtreme Gradient Boosting (XGBoost) is a supervised machine learning algorithm that provides an ensemble of weaker and simpler models to predict a target variable more accurately (Khan et al., 2022).
By using gradient boosting, variance and overfitting are reduced and there is a significant increase in the model robustness. This ensemble algorithm is suitable for both regression and classification tasks.
Since a classification algorithm is more appropriate for disease prediction than a regression one, XGBoost Classifier was used in the SageMaker platform.
SageMaker XGBoost has several strong points over the standalone XGBoost library, as the ones of SageMaker PCA algorithm, namely scalability, as the regular model can handle large datasets; integration with AWS services,

such as uploading training data to a S3 bucket; model tuning for optimal values of hyperparameters.

In Figure 3, several hyperparameters are set, such as the maximum depth of a tree or the number of classes which are two, represented in binary and it establishes the presence or absence of a cardiovascular disease in a certain patient.

```
Xgboost_classifier.set_hyperparameters(max_depth=3,
                                        objective='multi:softmax',
                                        num_class=2,
                                        eta=0.5,
                                        num_round=150)
```

Figure 3. XGBoost Classifier Hyperparameters
Source: own source

H. ***Integration of ML Model with Web Application***

The connection between the model and the web application is made once it has been trained, tested and ready for use. The integration is done with Boto3, an AWS SDK for Python programming language, which invokes the SageMaker endpoint and returns predictions based on patient's input data, as provided in Figure 4.

```
# session

session = boto3.Session(
    aws_access_key_id='AKIASK6QF24XODPI3DEI',
    aws_secret_access_key='Ss411SiueWF+hYs1nUJ+2xip1brdWy0xUMn+PI',
    region_name='us-east-1'
)

# get client instance
client = session.client('sagemaker-runtime',region_name='us-east-1')

print(type(client))

# invoke endpoint with input data
response = client.invoke_endpoint(
    EndpointName='sagemaker-xgboost-2023-04-28-09-26-22-062',
    Body=csv_data,
    ContentType='text/csv'
)

# convert the response from AWS service
# into Python object
predictions = json.loads(response['Body'].read().decode())
```

Figure 4. Endpoint Invocation and Prediction Retrieval
Source: own source

The doctor will receive a warning message on the screen that shows whether a patient belongs to a category of risk for cardiovascular disease or not. Furthermore, the incorporation of a machine learning model in the healthcare sector enhances the functionality of medical processes, helping the professionals to visualize data better and to make informed decisions based on real-time predictions.

RESULTS AND DISCUSSIONS

Considering the machine learning model, the results are based on the category of risk for whether a patient might suffer from a cardiovascular disease or not. The accuracy is based on each patient's medical data, such as age, height, weight, blood pressure. In this manner, the medical providers gain a granular understanding of the patient's health data, and it enables them to identify patterns and inconsistencies in the model. The accuracy of the model is computed using the following formula:

$$\frac{No.\ of\ correctly\ predicted\ labels}{No.\ of\ data\ points\ from\ dataset} \cdot 100 \qquad (1)$$

Another limitation is based on appointment scheduling, as the user is not shown on the screen the availability of a day and timeslot when a particular day and time from a certain department are chosen. This issue could be resolved with AJAX, which can update specific parts of a web page, without the need to reload the whole page.

Alongside the prediction of the category of risk for a cardiovascular disease, there have been several use cases implemented in this prototype:

- Sign-Up (for both patients and doctors), where the user enters personal information such as first name, last name, email address, as illustrated in Figure 5.

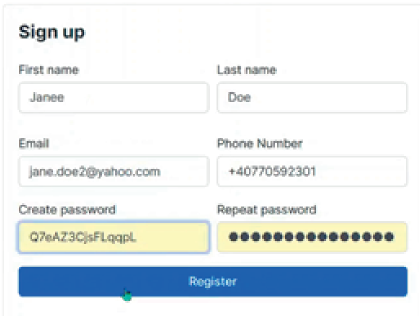

Figure 5. User Sign-up Interface
Source: own source

After the user presses the registration button, the information will be stored in the database and a success message will be displayed.
- Login (for both patients and doctors), allows the user to enter their credentials, which are the username and password.
After the user presses the login button, they will be redirected to their personal dashboard. Similarly, the Logout (for both patients and doctors) has been included in the application.
- Appointment Scheduling (patients only), as patients can book their appointment by completing the form from Figure 6.

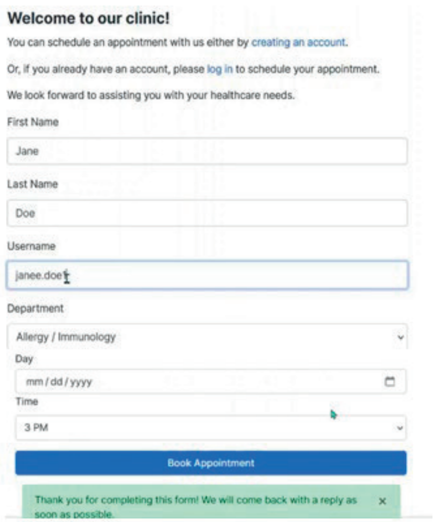

Figure 6. Appointment Scheduling Interface
Source:own source

- View personal details and appointments (patients and doctors), where the dashboard is as in Figure 7.

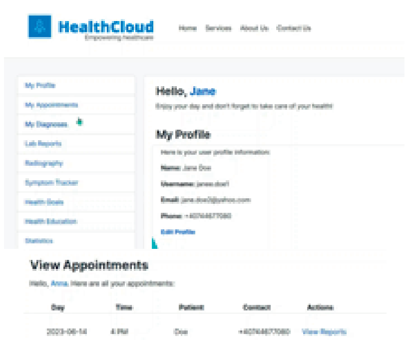

Figure 7. Personal Details and Appointments Interface
Source: own source

- View corresponding patients (doctors only), as doctors have an additional feature to view their patients' personal details, as in Figure 8.

Figure 8. Doctor's Interface for Patient Visualization
Source: own source

- Edit personal details (for both patients and doctors), where both patients and doctors can edit their personal details, that will be updated in the database, as in Figure 9.

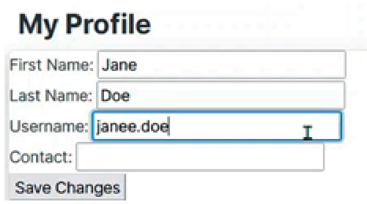

Figure 9. Edit Personal Details Interface
Source: own source

- Get disease risk feature, as after choosing the "Get disease risk" option from the patients view, a binary score will be shown on the screen which will establish the category of risk of the patient, as in Figure 10.

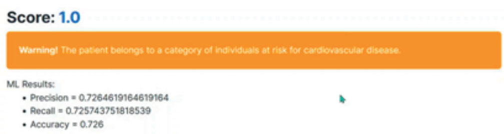

Figure 10. Get Disease Risk Feature Interface
Source: own source

CONCLUSIONS

The current paper highlighted the integration of Amazon Web Services (AWS) in healthcare management. Through conducting thorough research on various state-of-the-art models and comparing them with the proposed prototype, one can conclude that the project idea has proved its potential. The article has outlined the importance of digital transformation in healthcare, the challenges of chronic disease management, the potential of machine learning and AI, and the benefits of cloud hosting with AWS Amplify.

Improvements to the current work and future enhancements include first the fact that incorporating Amazon Rekognition for automated image analysis, such as chest X-rays, could greatly benefit early detection of pneumonia, leading to prompt intervention and better patient outcomes. This scalable AWS service supports telemedicine, allowing patients to easily upload radiographs for analysis, thus enhancing remote healthcare delivery. Secondly, the implementation of a Cloud bot using Amazon Lex presents an opportunity to create a virtual assistant in healthcare. This digital agent aims to simplify access to critical information, improve patient engagement, and provide 24/7 availability. Leveraging advanced natural language models, the bot can offer a conversational interface with speech and text capabilities, enhancing patient-provider interaction. Lastly, the extension of the current web application can include a Symptom Tracker mobile application. Tailored for patients with chronic conditions, this application can enable health data tracking and generate insights for self-management and remote monitoring. Utilizing Flutter as a cross-platform framework and Amazon Amplify for hosting, the Symptom Tracker application offers flexibility and scalability across different platforms.

In summary, while the current paper has laid the foundation for integrating AWS into healthcare management, there are opportunities for further enhancement and expansion. By leveraging emerging technologies and AWS services, the project can continue to evolve, addressing the ever-changing needs of the healthcare industry and ultimately improving patient care and outcomes.

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MILK YIELD AND PHYSICO-CHEMICAL COMPOSITION OF MILK OF ROMANOV SHEEP BREED RAISED IN THE FOOT-HILL AREAS OF BULGARIA

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Abstract

Romanov sheep breed was distributed in Europe more than 50 years ago. It is attracting more and more interest in Bulgaria because of its high fertility, but data on its milk productivity are scarce. The present study aims to determine the milk yield and physico-chemical composition of milk from Romanov sheep as it is the first of its kind in Bulgaria. 137 milk samples obtained from the 30th to the 120th day of lactation of 43 purebred Romanov ewes were analyzed. The average daily milk yield was 0.510 l, reaching 0.720 l as it gradually decreased during lactation. The milk yield for the studied period was 45.7 ± 3.52 l. The percentage content of milk fat increased with advancing lactation, as lactose showed insignificant changes. The protein indicator increased at the beginning, then it slowly decreased.

Key words: *milk yield, physicochemical composition, Romanov.*

INTRODUCTION

During the first weeks of life of newborn lambs, the main source of food is the mother's milk. They depend on the milk productivity of the mother, which is one of the main factors for the growth, development, health, and viability of the young organism (Lobkov et al., 2012).
In the first month of their life, the habituation to coarse fodder and the development of the proventriculus begins, and the most intensive growth of the animals is also reported (Ziangirova, 2020).
According to Radzik-Rant et al. (2017), sheep's milk contains biologically active compounds (enzymes, hormones, and vitamins) along with essential nutrients (proteins, fats, lactose, and total solids) that ensure the development of lambs and can influence the meat qualities. Breed, ewe age, feeding, and lactation period also influence milk composition (Atti et al., 2006; De La Fuente et al., 2009; Rozbicka-Wieczorek et al., 2015).
Tsochev et al. (2018) reported that milk yield in sheep is a heritable trait with high variation, both between breeds and between individuals within a breed, with a breed effect demonstrated on lactation and milk quality (Skoufos et al., 2017; Thomas & Haenlein, 2017; Moatsou & Sakkas, 2019).
According to Rozbicka-Wieczorek et al. (2015), the content of the main components in milk is determined by genetic and environmental factors. The milk of the dairy sheep breeds has a lower fat and protein content than the milk of breeds of other trends. Insignificant differences in milk composition were found in different meat-producing breeds (Peniche et al., 2015).
Establishing the quality of the milk and the higher milk yield of the ewes is a prerequisite for a higher increase in the rearing of lambs for meat production. There is a positive correlation between lamb growth and the amount of milk secreted by dams in early lactation (Wohlt et al., 1981).
Sheep's milk contains more total solids, protein, fat, and calcium. It follows that the calories in sheep's milk are almost twice as much as in cow's and goat's milk (Lesnovska, 2014).
Lobkov et al. (2012) found that under favorable feeding conditions high milk yield is ensured for the Romanov ewes, therefore they can raise two, often three lambs without giving them milk replacer.
According to Tekel et al. (2020), the Romanov breed has a high milk yield, which is sufficient not only to feed a large number of offspring, but also for the production of dairy products (Kostylev et al., 2015), but the data that

confirm it are limited. In Bulgaria, the milk of the Romanov sheep breed was studied by Tsochev (1983), and he reported data only on the milk yield of the first and second lactation and the content of milk fat and protein substances. The limited amount of information on milk yield and quality of milk from the Romanov breed necessitated the present study, which aims to determine the milk yield and physico-chemical composition of Romanov sheep and is the first of its kind in the contemporary conditions of Bulgaria.

MATERIALS AND METHODS

The study was conducted with 43 pure-bred Romanov ewes raised in the Scientific-Experimental Base at the Research Institute of Mountain Stockbreeding and Agriculture (RIMSA) of Troyan. The sheep are of different ages and stages of lactation. 15 ewes were in the first and second lactation, 15 ewes in the third lactation, and 13 ewes between the fifth and eighth lactation.

During the study period, the animals were kept in a goat shed and fed according to norms for lactating ewes. The daily ration included bulk feed of good quality meadow hay, oat straw, and concentrated feed containing corn, wheat, sunflower meal, and vitamin-mineral premix. Animals had continuous access to water. During the first ten days after parturition, the lambs together with their mothers were raised in individual boxes, then they were gathered in a group box, and between the 20th and 30th day, the lambs were separated from their mothers.

The 90-day milk yield of the ewes was determined by individual hand milking at each milking check. The lambs were separated from their mothers 12 hours before the control milking, and the amount of milk produced by each ewe was measured. To determine the milk yield for the entire control day, the milk yield was doubled. The duration of the control period averaged 30 ± 3 days. Milk yield for a control period is the product of the amount of milk determined on the control day and the number of days in the control period. The 90-day milk yield is the sum of the amount of milk from the individual control periods.

To determine the quality of milk, individual samples were placed in containers with a capacity of 50 ml. Immediately after receiving, the milk samples were examined in the Analytical Laboratory of RIMSA to determine the main physicochemical parameters such as milk fat content (%), protein (%), lactose (%), solid-not-fat (%) and total solids (%) with milk analyzer "Laktoskan LW". The statistical processing of the data was conducted using the statistical package Data Analysis, Excel 2021, Microsoft.

RESULTS AND DISCUSSIONS

Milk yield is influenced by several genetic and non-genetic factors, such as feeding, animal movement, lamb presence, and udder stimulation during lactation (Kostylev et al., 2015), as well as animal temperament (Stoycheva et al., 2014).

The average daily milk yield for the 90-day study period was 0.510 l. For the researched herd, the peak of the average daily milk yield was between the 40th and 50th day with 0.720 l. A significant gradual decrease in the average daily milk yield was reported as the days of lactation of the ewes progressed (P<0.05) (Figure 1).

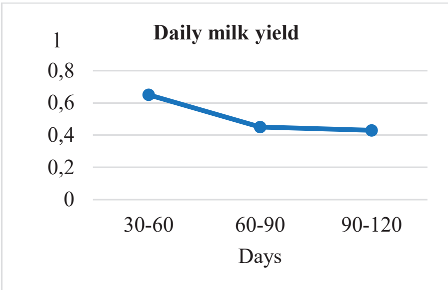

Figure 1. Dynamics of average daily milk yield for 90 days

The average daily milk yield in the present study was 0.184 l more than that reported by Boylan and Sakul (1988) for Romanov ewes with a daily milk yield of 0.326 l. In contrast to the present results, Kostylev et al. (2015) reported a higher milk yield, which reached 1550 g on the 10th day. After that, it gradually decreased and by the end of lactation, the daily amount of milk reached 100-150 g, and the milk yield was determined by weighing the lambs. The dynamics of changes in the amount

of average daily milk yield reported by Boylan & Sakul (1988) and Kostylev et al. (2015) are consistent with our results.
The results of the present study do not correspond with those obtained by Magomedov (1976), who found an average daily milk yield of 1.661 to 1.758 l.
In contrast to our results, Magomedov (1976) and Kostylev et al. (2015) reported the milk yield of sheep by weighing the lambs before and after suckling.
During the studied 90-day period, the average amount of milk was 45.7±3.52 l.
Kutlucaa et al. (2011) reported that in F1 crossings Morkaraman x Romanov, the milk yield was 74.2 kg, and in F1 crossings Awassi x Romanov it was 104.3 kg.
The ewes with one lamb (Figure 2) had a 38.7% lower milk yield than the ewes that gave birth to twins (Figure 3). The ewes with triplets (Figure 4) had a 24.6% lower milk yield than the ewes that gave birth to twins (Figure 5).

Figure 2. Romanov ewe with a single lamb (own source)

Figure 3. Romanov ewe with twins (own source)

Figure 4. Romanov ewe with triplets (own source)

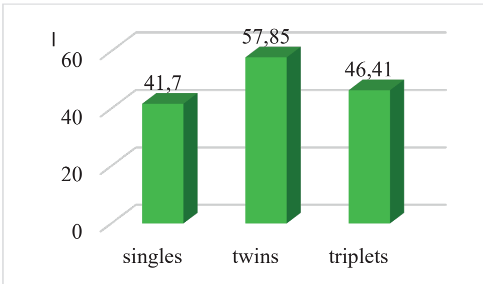

Figure 5. Milk yield of ewes according to type of parturition

Magomedov (1976) cited by Tsochev (1983) found 157.8 l for the first and 190 l for the second lactation of ewes with a delicate constitution, and for animals with a rough constitution, respectively, 149.8 and 167.2 l, which does not correspond to our study.
In contrast to our results Kostylev et al. (2015) reported that a ewe with one lamb gave 97 kg of milk, when two litters were lambed, the amount of milk for lactation was 155.4 kg, and when triplets were born it was 161 kg.
According to Kostylev et al. (2015), milk production is affected by the age of the mother, as young animals in the first and second lactation produce less milk than adults. The milk yield of the ewes increases until the 5th or 6th lactation, after which it gradually decreases. Assan (2020) reported that milk yield was higher in multiparous beef breeds.
In the present study, the daily milk yield in the 3rd lactation of ewes was 0.120 l more than the 1st and 2nd lactation of ewes (Figure 6).
The milk yield of the sheep in the 1st and 2nd lactation was 39.25 l for 90 days. The milk productivity of the sheep in the 3rd

lactation was 50.62 l or 29% more than the previous group.

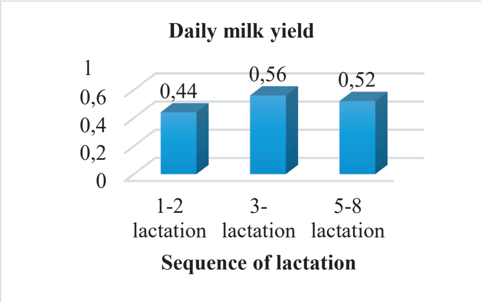

Figure 6. Daily milk yield according to the sequence of lactations

Since the Romanov breed is precocious (Gatsiev, 2015), during the first lactations the animals have not completed their growth and development, therefore large amounts of energy are used for their growth instead of milk production (Bancheva et al., 2023). In the group of ewes between the 5th and 8th lactation, the amount of milk was 46.48 l, which was 8.9% less than the ewes in the 3rd lactation (Figure 7).

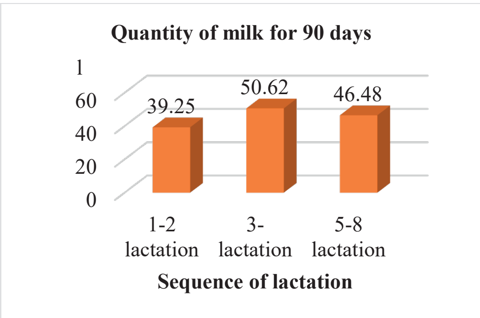

Figure 7. 90-day milk yield according to the sequence of lactations

In animals that have completed their growth, the maximum number of offspring in one parturition is observed and the highest milk yield is established (Ziangirova, 2020).
Tsochev (1983) found that animals in the 1st lactation had a milk yield of 87.6 l, as it was 107.3 l in the second lactation for 100 days, and the amount of daily milk was the highest in the first month.
Smirnova (1954) cited by Arsenyev et al. (2011) reported that for 100-day lactation, the milk productivity of Romanov sheep with one lamb was 97.2 kg, with two lambs was 120 kg, with three lambs was 153 kg and with four lambs was 169.1 kg, which is not confirmed in our results.
Erokhin et al. (2005) found that according to age, body weight, constitution, and the size of the mammary papillae, the largest daily amount of milk was obtained by the mothers of the 5th or 6th lactation with 956 g, after which the milk yield decreased.
Lobkov et al. (2012) reported that ewes in the 3rd lactation released 142 kg of milk, 151.9 kg in the 4th, 170.8 kg in the 5th, 169 kg in the 6th, and 149 kg in the 7th and 8th lactations. The study mentioned above shows clearly that sheep in the 5th lactation gave the largest amount of milk.
In the Romanov herd examined in the present study, the ewes in the 3rd lactation had the highest milk yield, but they occupied the largest share of the herd. Sheep between the 5th and 8th lactation were collected in a group where the recorded amount of milk was lower since the group also included ewes in the 7th and 8th lactation.
In contrast, Smirnov (1953) quoted by Tsochev et al. (2018) found the highest milk yield in the 2nd lactation, respectively in the 1st lactation the milk yield was 111.48 l, 157.71 l in the 2nd, and 111.37 l in the third.
The lactation length, as well as the physico-chemical composition of milk, are genetically determined by the breed. However, they are significantly affected by the stage of lactation, the nutrition of the ewes, the body and health condition of the ewe, and the environmental factors of the respective economic year (Nudda et al., 2014; Skoufos et al., 2017). The decrease in milk fat during lactation may be due to increased milk secretion and reduced fat mobilization from adipose tissue (Chilliard et al., 2003).
Changes in the physico-chemical composition of milk during lactation periods are presented in Table 1.
As lactation progresses, fat content increases reliably from the 90th to the 120th day ($P<0.05$). Protein and lactose indicators do not change significantly during lactation.
The solid-not-fat remains within narrow limits, and at the beginning of the study, it was 0.15%

more than the previous period. A reliable gradual decrease of the total solids index with advancing lactation of the ewes ($P<0.05$) is reported, which is associated with the decrease of milk secretion (Table 1).

Table 1. Main indicators of milk

	Milk fat, %	Protein, %	Lactose, %	SNF, %	Total solids, %
30-60 day	$6.75\pm0.23^{b*}$	5.03 ± 0.04^{ns}	4.71 ± 0.04^{ns}	10.57 ± 0.09^{ns}	17.32 ± 0.22^{b}
60-90 day	7.33 ± 0.21^{ab}	4.95 ± 0.03^{ns}	4.64 ± 0.03^{ns}	10.41 ± 0.06^{ns}	17.75 ± 0.21^{ab}
90-120 day	7.87 ± 0.21^{a}	4.96 ± 0.04^{ns}	4.64 ± 0.04^{ns}	10.42 ± 0.08^{ns}	18.29 ± 0.20^{a}
LSD 0.05	0.60	0.10	0.10	0.22	0.60

Note: *Means followed by the same letters are not significantly different; ns - No statistical proof.

When examining the 28th and 56th day of lactation of the Dorset, a meat and wool-producing sheep breed, Wohlt et al. (1981) found higher results on the 28th and 56th day than the mentioned above, respectively the milk fat content was 11.4 and 13.2%, the protein was 4.9% and 4.6%, lactose was 5.3 and 4.5% and total solids were 22.5 and 23.1%.

Our results are close to Skoufos et al. (2016), who studied three dairy breeds with milk fat between 6.1% and 8.5%, protein from 5.43% to 5.95%, lactose from 4.5 to 4.64 and total solids from 19.51 to 20.04%.

Boylan and Sakul (1988) indicated average values of milk fat content of 6.4%, protein was 6.1, lactose was 4.8, and total solids were 18.1% for the Romanov sheep breed. The average milk fat content in the present study is 0.92% higher, the protein is 1.12% lower, for the lactose indicator the difference is insignificant, and the percentage of total solids is 0.32% higher than our results.

Kostylev et al. (2015) studied a Romanov herd and recorded milk fat content and protein of 6.3, respectively; 5.23% at the beginning of lactation, 6.15; 5.13% in the middle and 7.35%; 6.20% at the end of lactation, for lactose at the beginning of lactation reported 5.15%, 4.7% in the middle and 4.5% at the end of lactation.

The total solids at the beginning were 17.9 ± 0.04, in the middle 17.1 ± 0.3 and 19.2 ± 0.4 % at the end of the lactation period. The milk fat content, in the present study, at the beginning of lactation was 0.44%, in the middle was 1.18%, and at the end of lactation was 0.52% more. The protein was 0.2% lower at the beginning, 0.18% lower in the middle and 1.24% lower at the end of lactation.

The lactose level was 0.44% lower at the beginning of lactation in our results, but in the middle and at the end of lactation the values were similar.

According to Tsochev (1983), a herd of Romanov sheep in the 1st lactation had an average milk fat content and protein of 7.85 and 5.6%, respectively, for the 2nd lactation the fat content was 7.78% and 5.4% protein, as the data correspond to our data.

In a study in Poland of a local breed of sheep, Rozbicka-Wieczorek et al. (2015) and later Radzik-Rant et al. (2017) confirmed the dynamics of physicochemical parameters found in the present study.

CONCLUSIONS

A significant gradual decrease in the average daily milk yield of ewes was reported as the days of lactation progressed ($P<0.05$).

Mother ewes in the 3rd lactation had a higher milk yield than ewes in the 1st and 2nd lactation.

In the course of lactation, milk fat content, and dry matter increased reliably, whereas the values of protein, lactose, and solid-not-fat did not change significantly.

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THE REPRODUCTIVE PERFORMANCE OF COWS SPECIALIZED FOR MILK PRODUCTION IN THE PROCESS OF ADAPTATION TO NEW LIFE CONDITIONS

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Abstract

The reproductive performances (SP - service period, CI - calving interval, CCR - Reproductive capacity coefficient) of Jersey (Js), Holstein (HL) cow populations of French, Dutch and local selection were studied, in the period of adaptation to the new operating conditions. It was established that the most significant deviations from the optimal physiological value of SP and CI are found in the Js breed (SRL "Agro-Gogu") (+104.9 and +113.4 days) and in the local selection HL population (SRL "Gomets Efrem") (+78.2 and 88.6 days). In the dynamics of lactation, the duration of SP and CI, regardless of the animal husbandry, but also of the breed, is far above the optimally allowed values. The most favorable results are attested to heifers first calving of the French HL breed. The CCR reached the maximum value in cows HL of local selection, from the second lactation, followed by the Js population. The analyzed data reveal that the adaptive response of the animals to the new conditions is very slow, with breed differences and intrabreed variability.

Key words*: adaptation, cows, imported animals, local selection population, reproductive performance.*

INTRODUCTION

The revitalization and improvement of herds of cows specialized for milk production is achieved through the import of live animals, semen, embryos etc. When importing animals from abroad, we must strive not only to obtain high productivity from them, but also to ensure their long-term use in order to obtain as many young animals as possible for breeding. Dysregulation of the reproductive abilities of cows leads to lower birth rates and lower milk productivity (Deshevykh et al., 2016).

Sulyga & Kovaleva (2010) established that in some animals, acclimatization occurs quickly, without special changes in the physiological state, while others require months and even years, as environmental factors affect imported animals differently. Filinskaya et al. (2020) claims that adult animals acclimatize worse than young animals that were raised in new conditions for the breed. According to Evstigneev (2010), the acclimatization of imported animals to new natural and climatic conditions is never complete and occurs only in the first or second generation and, in some cases, may not be observed at all. Therefore, the successful introduction of productive animals in new conditions is ensured if there is sufficient information about the growth and productive and farrowing status of the animal breeding sector of the exporting country (Shabalina, 2011; Korotkikh et al., 2019) reports that for domestic animals, the distribution area can be expanded due to the creation of maintenance and feeding conditions corresponding to their natural properties. Adaptive reactions in the body can be caused by various factors and can be expressed in changes in growth and development, the level of milk productivity, the morphological and functional properties of the udder and the reproductive abilities of cows (Vostroilov et al., 2019; Amaritii & Maciuc, 2023). Good reproductive qualities in new climatic and economic conditions often indicate the normal course of the acclimatization process in these animals (Usenkov et al., 2011).

Since about 70% of the herd intended for milk production consists of imported animals, and taking into account the pedoclimatic conditions of the Republic of Moldova, which are different from one area to another, in order to develop the strategy of selection and improvement works with these contingencies, the study of the reproductive qualities of the imported animals in the process of adaptation to the new living conditions and of their offspring is a very urgent and indisputable problem. The reference objective focused on the study of the main reproductive indicators in cattle of different breeds, specialized for milk production in the process of adaptation to new living conditions.

MATERIALS AND METHODS

It was experimented with Holstein breed cow populations of different origin from the zootechnical units: SRL "Doksancom", v. Tomai, Chiadâr-Lunga, the Holstein breed of Dutch origin (HLD); SRL "Gomerts Efrem", Comrat municipality, the local selection Holstein breed (HLLS); SRL "Holstein", v. Roshcani, Anenii Noi district, the Holstein breed of French origin (HLF) and the population of Jersey (Js) cows SRL "Agro-Gogu" v. Ruseni, Anenii Noi district.

To characterize the reproductive capacity of cows, the following indices were evaluated: SP - length of service period, CI - interval between calvings, CCR - coefficient of reproductive capacity.

In order to establish the interrelationships between SP, CCR and milk productivity indices, the direction and magnitude of the correlation coefficient (r) was calculated between them and: the amount of milk per total lactation (SP- and CCR-MTL); the amount of milk during normal lactation, kg (SP- and CCR-MNL); overall fat, kg (SP- and CCR-OFNL).

The material for the research served the data of zootechnical accounting (card form T-2) and the information database of the farms.

The coefficient of reproductive capacity (CCR) of cows was calculated according to Èisner (1978) (1).

$$CCR = \frac{365}{CI} \qquad (1)$$

The aim was to obtain position estimators: the arithmetic mean - $\bar{X}$; the standard deviation of the mean - Sx; the coefficient of variation - Cv; the correlation coefficient - (r).

The authenticity of the obtained results was verified according to the Student criterion - td.

Statistical processing and biometric analysis of the obtained data were carried out according to the generally accepted methods of variation statistics (Grosu, 2005) using the MS Excel-2010 analysis software package.

RESULTS AND DISCUSSIONS

The breeding efficiency of dairy cattle is largely determined by the reproductive intensity of the female herd, which has a direct impact on milk productivity.

Among the factors that determine milk productivity in cows, along with feeding conditions, maintenance, genetic potential an important role is occupied by the reproductive state of the female herd. The main indicators of the state of reproduction of the herd are: the length of the service period, the length of the interval between calvings, the reproductive capacity coefficient of the cows, the determining factor being the service period (Lyashuk & Mikhailova, 2016). In Table 1, we present the results of some reproductive indices (SP - service-period and CI- interval between calvings) in Holstein cows, of different origins, operated in different zootechnical units.

Table 1. The characteristic of the service period and the interval between calvings in the populations of cows specialized for milk production operated in different zootechnical units

Specification	The service period, *days*			Tne calving interval, *days*		
	$\bar{X} \pm S_x$	±with the optimal value allowed (90), days/%	Cv, %	$\bar{X} \pm S_x$	± with the optimal value allowed (365), days/%	Cv, %
SRL "Doksancom" (HLD)	129.2±7.20***	+39.2/43.5	52.3	411.3±7.28***	+46.3/12.7	15.3
SRL "Gomerts Efrem" (HLLS)	168.2±16.19	+78.2/86.7	63.1	453.6±16.13	+88.6/24.3	23.3
SRL "Holstein" (HLF)	138.0±12.18***	+48.0/53.3	7.6	422.5±12.10***	+57.5/15.8	7.2
SRL "Agro-Gogu" (Js)	194.9±12.03	+80.8/89.8	59.8	45.5±18.77	92.5/25.3	26.4

***P<0.001.

The obtained results demonstrate that in all cow populations, the duration of the service period essentially prevails the maximum allowed value (90 days) within the limit of 39.2 days for

HL cows, Dutch origin from SRL "Doksancom" and 104.9 days for Jersey cows, from SRL "Agro-Gogu". Close to the minimum extreme of deviation, from the physiologically optimal value, the French HL population from SRL "Holstein" is placed with a difference of 48.0 days and towards the maximum extreme of deviation is the HL local selection population from SRL "Gomerts Efrem" with a difference of 78.2 days.

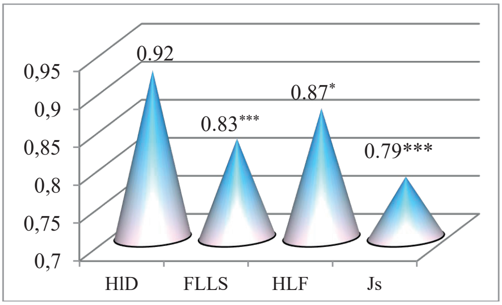
The comparative study of the value of the service period according to the origin of the animals and the animal husbandry shows statistically authentic differences lower by 65.7 days (td=4.7. P<0.001) in the herd of SRL "Doksancom" (Holstein breed of Dutch origin) and by 56.9 days (td=3.3. P<0.001) in the livestock of SRL "Holstein" (Holstein breed of French origin), compared to the Jersey population exploited in SRL "Agro-Gogu". Based on the analysed data, we mention that the terms of fertile insemination of cows, in all cases, exceed the maximum allowed limit, the latest being found in the herd of cows from SRL "Agro-Gogu".

Regardless of the origin of the animals, but also of the animal husbandry, a great degree of heterogeneity is attested within the herds (52.3-64.4%) in terms of the duration of the service period. The highest coefficient of variation is in the population of Jersey cows from SRL "Agro-Gogu". In the Holstein breed, French origin from SRL "Holstein", the herd is much more homogeneous, the coefficient of variation is 7.2%. These data demonstrate that the service period is mainly influenced by external factors (technological, pedoclimatic).

The duration of the interval between calvings (Table 1) in the investigated populations exceeded the optimal term (365 days), depending on the livestock farm, but also on the origin of animals, the differences are within the deviation limit of 46.3-113.4 days, forming the series: Holstein of Dutch origin (46.3 days) < Holstein of French origin (57.5 days) < Holstein of local selection (88.6 days) < Jersey (113.4 days).

The interval between calvings is maximum in the cow population Jersey breed, from SRL "Agro-Gogu" (478.4 days). Compared to the Holstein breed of Dutch origin from SRL "Doksancom", it is higher by 67.1 days (td=4.7. P<0.001,) and by 55.9 days (td=3. P<0.001) to the Holstein population of French origin from SRL "Holstein". In the rest of the cases, both regarding the length of the service period and the intervals between calvings, the discrepancies are statistically inauthentic.

In practice, the coefficient of reproductive capacity of cows is an essential economic index that reflects the state of reproduction of the herd and directly depends on the duration of the interval between calvings, which in turn depends on the service period. The service period also conditions the duration of lactation, but also the birth rate per population, as a whole. In Figure 1 we present the dynamics of the coefficient of reproductive capacity of Holstein cow populations, of different origins.



**P<0.05. ***P<0.001.

Figure 1. Characteristic of the coefficient of reproductive capacity in cow populations of breeds specialized for milk production

The optimal level of the coefficient of reproductive capacity of cows is equal to one unit and depends on the length of the interval between calvings. The value of the coefficient of reproductive capacity at the cow populations differ both according to breed, origin, and livestock holding, occupying the range 0.92-0.78.

The best result (0.92) is found in the population of Holstein cows, of Dutch origin, from SRL "Doksancom". The most significant difference (0.14) (td=5.9. P<0.001) was established between the herds of Holstein cows from SRL "Doksancom" (southern pedoclimatic zone) and the population of Jersey cows, SRL "Agro-Gogu". At the same time, in comparison with the Holstein populations of French origin, SRL "Holstein" (central pedoclimatic zone), a genuine statistical difference of plus 0.05 (td=2.2; P<0.05) is found in favor of the first.

Also, statistically authentic discrepancies were elucidated in the Holstein cow herds of local selection from SRL "Gomerts Efrem", the results being lower by 0.09 (td=3.9; P<0.001), compared to the Dutch population from SRL "Doksancom", both being exploited in the southern pedoclimatic zone, but in different zootechnical units. At the same time, the value of the coefficient of the reproductive capacity of cows in the herd of SRL "Holstein" in which the Holstein breed of French origin is exploited, is significantly higher by 0.08 (td= 5.2; P<0.001), compared to the population of SRL "Agro-Gogu", both located in the central pedoclimatic zone of the country.

In practice, the coefficient of reproductive capacity of cows is an essential economic index that reflects the state of reproduction of the herd and directly depends on the duration of the interval between calvings, which in turn depends on the service period. The latter also conditions the duration of lactation, but also the birth rate per population, as a whole (Table 2).

Table 2. Duration of lactation and the birth rate, in cow populations of breeds specialized for milk production

Specification	Duration of lactation, *days*	The birth rate, *calves*
SRL "Doksancom" (HLD)	356.2±7.14***	90.3±1.53***
SRL "Gomerts Efrem" (HLLS)	381.9±16.36*	81.2±2.15
SRL "Holstein" (HLF)	362.9±2.41***	86.4±0.43**
SRL "Agro-Gogu" (Js)	425.9±12.7	78.7±2.01

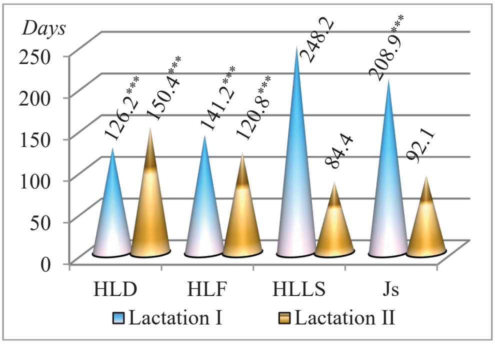
*P<0.001. **P<0.001. ***P<0.001.

Lactation duration in all cow populations exceeds the physiologically optimal value (305 days). The closest to the allowed duration was found in the population of the Holstein breed of Dutch origin, the gap being 51.2 days, 57.9 days in the population Holstein of French origin, 76.9 days in the Holstein local selection population and 129.9 days in the Jersey breed cows.

Depending on the origin of the animals, in the Holstein population of Dutch origin, vis-à-vis the Jersey breed population, the difference is - 69.7 days (td=4.6; P<0.001), - 63 days (td=4.9; P<0.001) in French Holstein and - 44 days in the local selection population (td=2.1; P<0.05).

Based on the analysed data, the number of calves obtained at 100 cows per year is statistically genuine differences, compared with Jersey population. The best results in Dutch Holstein cows (td=4; P<0.001). A genuine statistical difference was also evident in the Holstein population of French origin (td= 2.6; P<0.001).

In connection with the data from the profile literature according to which the adaptation process can occur in the first or subsequent generations, we studied reproduction indices in the dynamics of lactations. In Figure 2 we show the characteristic of the service period in the dynamics of lactations.



***P<0.001.

Figure 2. The characteristic of the service period in the dynamics of lactation in cow populations from different breeds specialized for milk production

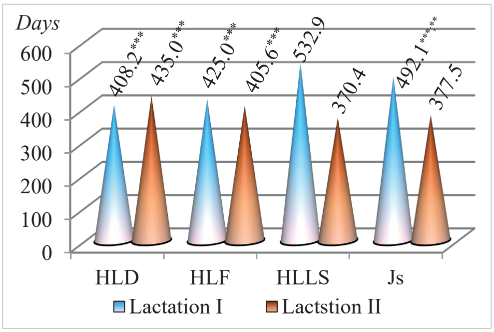
The duration of the service period, regardless of the order number of the lactation, essentially exceeds the optimal value allowed (90 days) except for the herd of cows, the second lactation, Holstein breed local selection from SRL "Gomerts Efrem" located in the southern pedoclimatic zone.

Comparing the results established for cows in the first lactation, extreme values of the service period are found in Holstein cows of local selection, being higher by 122 days (P<0.001, td=6.1), compared to the Dutch HL herd from SRL "Doksancom", the same southern pedoclimatic zone and with 107 days (td=5.6. P<0.00) with the result established in the Holstein, French population exploited in the central areas of the country.

The dynamics of the service period in the Holstein contingent of local selection from second lactation, the result is diametrically opposite to that established at the first lactation, the duration being the shortest. The differences are statistically significant lower by 66 days (td=4.5; P<0.001) vis-à-vis the result recorded in the Dutch Holstein population and by 36.4 days (td=4.4; P<0.001) in the French Holstein population.

The duration of the service period in different breeds shows that in Jersey cows it is statistically significantly higher by 81.7 days (td=5.5; P<0.001) and by 67.7 days (td=3.6; P<0.001) compared to Dutch and French Holstein populations, respectively.

Significantly greater differences were established in the Dutch Holstein herd by 58.3 days (td=3.3; P<0.001) and in the French Holstein population by 28.7 days (d=3.4; P<0.001), compared to Jersey cows. The duration of the interval between calvings in the dynamics of lactations, in all the populations included in our investigations, are higher compared to the physiologically optimal value (Figure 3).



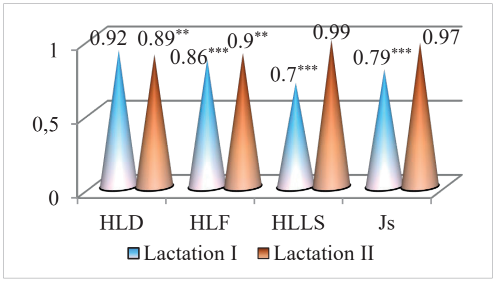
**P<0.001. ***P<0.001.

Figure 3. The characteristic of the calving interval in the dynamics of lactation in cow populations of different breeds specialized for milk production

In first lactation cows, the largest gap was established in the population of local selection cows, exceeding with statistically genuine differences of 124.5 days (td= 6.9; P<0.001) (SRL "Doksancom") and 107.9 days (td=6.5; P<0.001) (SRL "Holstein"). Statistically greater genuine differences in these populations were also established compared to the Jersey breed population from SRL "Agro-Gogu" 83.9 days (td=5.7; P<0.001) and 67.1 days (td=5.0; P<0.001).

The second lactation contingents show a similar trend to the situation in the case of the service period. The best result was demonstrated by the local selection of the Holstein population. In relation to the Dutch Holstein and French Holstein herds, differences of 64.6 days (td=4.4; P<0.001) and 35.2 days (td=4.2; P<0.001,) are found, respectively. Statistically smaller true differences of 57.1 days (td=2.8; P<0.01) and 86.5 days (td=5.4; P<0.001) in these latter two populations are also attested compared to the result established in the Jersey breed population.

Next, we present the dynamics of the coefficient of utilization of the reproductive capacity of herds of cows from specialized breeds for milk production, corresponding to the order number of the lactation (Figure 4).



**P<0.001. ***P<0.001.

Figure 4. Characteristic of the coefficient of reproductive capacity, in the dynamics of lactation, in cow populations of breeds specialized for milk production

From the analysed data, it is evident the influence of the service period on the duration of the interval between calvings, which in turn is directly reflected on the coefficient of utilization of the cows' reproductive capacity.

Comparing the results obtained in the first lactation, the best coefficient of utilization of the reproductive capacity of the female herd is found in the Holstein breed population of Dutch origin exploited in the southern pedoclimatic zone (SRL "Doksancom"), while among the herds in the second lactation, the superiority is held by the Holstein cows of local selection, from SRL "Gomerts Efrem", located in the south of the country, followed by the contemporaries of the Jersey breed exploited in the central area.

Based on the results recorded on two normal lactations (305 days), we note that the reproductive capacity coefficient of the female population, regardless of breed, provenance or pedoclimatic zone, is better in cows from the second lactation.
In the following we present the interrelationships between SP, CCR and milk productivity indices in the cow populations included in the study (Figure 5).

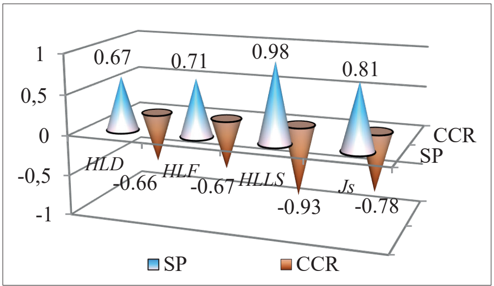


Figure 5. Relationship between SP, CCR and milk productivity for total lactation, in cow populations of breeds specialized for milk production

The analysed results attest to the presence of a high and very high positive correlation between service period and milk productivity on total lactation. Under the same maintenance and exploitation conditions, the magnitude of the correlation coefficient between reproductive capacity and milk productivity on total lactation remains strong and very strong, over time which direction changes diametrically opposite - negative, regardless of the animal husbandry or the origin of the animals.
Similar results can be seen in the case of milk productivity in normal lactation (Figure 6), only changing the magnitude of the correlation coefficient from very weak to weak.

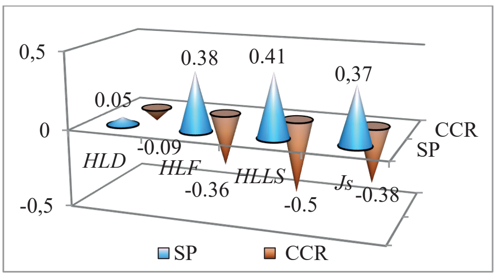


Figure 6. Relationship between SP, CCR and milk productivity for normal lactation, in cow populations of breeds specialized for milk production

Figure 7 reflects the direction and magnitude of the correlation between reproductive and overall fat on normal lactation.

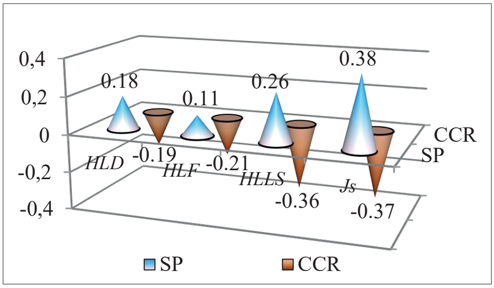


Figure 7. Relationship between SP, CCR and overall fat for normal lactation, in cow populations of breeds specialized for milk production

Between the overall fat and reproductive indices (SP and CCR) the presence of the correlative link was established, predominantly weak in magnitude, as a positive in the case of the service period and negative to the coefficient of use of the reproductive capacity of the mother herd.
The results obtained in our study have an ambiguous character. On the one hand, it confirms the results reported by Usenkov (2013), who, studying the economic, biological peculiarities and the acclimatization capacity of heifers imported from Germany, found that heifers of the Holstein breed had difficulties acclimatizing in the local conditions.
On the other hand, it rejects the view that even after the first calving they had high rates for all the basic economically valuable characteristics and practically all fell within the requirements of the target standards for native animals, which in our case, was not observed in none of the investigated populations, in the first lactation.
At the end of the analysis of the experimental results, we generalize that the indices that reflect the functionality of the reproductive system in cow populations imported from abroad, but also in the local selection population, did not fall within the requirements of the target standards. According to the value of the reproduction indices in the local selection population, as a whole in the population, compared to the imported animals, no adaptive reactions are attested for now. These being significantly higher with the result

established for the population of Holstein cows of Dutch origin exploited in the same southern pedoclimatic zone, and also lower results are attested for them and compared to the result established for Holstein cows of French origin, located in the central pedoclimatic zone of the country.

Thus, in dairy cattle breeding of the Republic of Moldova, when solving problems related to reproduction, it is necessary to apply an integrated approach, using modern genetic material, advanced technologies, including improving the conditions of feeding and keeping dairy cows.

CONCLUSIONS

The functionality of the reproductive system in cow populations imported from abroad, for now, does not fall within the requirements of the target standard, showing variations depending on the breed and their origin, but also of the technological and pedoclimatic conditions in which the animals are exploited.

The most significant deviations from the optimal physiological value of SP and CI can be seen in the Js breed (SRL "Agro-Gogu") (+104.9 and +113.4 days) and the local selection HL population, (SRL "Gomerts Efrem") (+78.2 and 88.6 days).

In the dynamics of lactation, the duration of SP and CI, regardless of the zootechnical unit, breed and its origin, are far above the optimally allowed values. The most favorable results are attested to primiparas (I-st lactation) of the French and Duch Hostein breed. For cows in the second lactation we mention the HL breed population of local selection. CCR reached the maximum value in cows HL of local selection.

The adaptive response of animals to the new conditions is very slow, with breed differences and intrabreed variability.

The successful introduction of productive animals in new conditions is ensured if there is sufficient information about breeding technology, productive status and also the level of selection and improvement of the animal breeding sector of the exporting country.

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STUDY ON QUANTITATIVE INDICATORS OF RAWHIDE IN SELECTED BEEF COWS AND COMBINED CATTLE BREEDS

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Abstract

The structural changes made in the cattle breed composition during the last 30 years in Bulgaria, along with studies on milk and meat productivity, have required the study of hides as a strategic raw material for the light industry. The relative weight, percentage of hide from the live weight, size, surface area, and thickness in certain sections of cull cows excluded from breeding for various reasons, from the following breeds were studied: Limousin, Galloway, Normande cattle, Bulgarian Red Cattle. Significant breed differences were observed in the examined hide parameters. The best represented in the space, as a spread of the configuration, was the raw hides obtained from Normande cattle (498.29 dm^2), followed by Bulgarian Red Cattle (461.23 dm^2). The worst indicators were found in Galloway (388.75 dm^2) (p <005)

Key words: *cows, combined breeds, hide, thickness, surface area, weight.*

INTRODUCTION

People have used animal hides for various purposes since the Paleolithic period. The production of leather goods is a century-old industry. Once the skin is torn from the animal's body, it is highly susceptible to microbial agents because it is rich in proteins and lipids (Nadal-Molero et al., 2023)

Cattle hide skinned from the animal is the main raw material for the leather, fur, and footwear industries, as well as for the production of souvenirs.

Cattle hide is the most common material of animal origin. It accounts for about 65% of the world leather market, as a waste product of the meat processing industry. Hides are limited in size, their quality is highly variable, the supply chain is low margin, with a highly fragmented value chain, and the production time is very long (Tubb, 2022).

The changes made in the cattle breed composition in Bulgaria in recent years suggest studying not only milk and meat productivity but also conducting studies on hides as raw material.

Skin performs basic vital functions in the body, such as protective, regulatory, and excretory. According to its condition and the shine of its hair coat, it is possible to predict the health status of cattle. The skin makes up about 7-9% of the live weight of the animal (Kibkalo & Zhersbilov, 2009; Badakhov, 2011).

Combined cattle breeds, which have a relatively large live weight and heavy and thick skin, usually reach about 35 kg skin weight and more, with thickness in the controlled areas from 4 to 4.5 mm (Badakhov, 2011; Kibkalo et al., 2014; Kozyr, 2018).

To characterize the hide qualities in various live cattle in practice, and in particular in adult cows, after the third calving and older, before slaughter are examined: the thickness of the skin fold of the front left hocks and the thickness of the skin in the middle on the last rib. The data obtained from seasonal measurements show that in Simmental breed the type, constitution, and season of production are essential for the quality of the hide (Panin, 2015; Nezavitin, 2015).

According to Adzinova & Mambetov (2018), the weight, surface area, sameness, and defects of cattle hide depend on sex, age, breed, and live weight, as well as on feeding conditions and technological parameters of rearing.

The objective of the present study was to examine, compare, and analyze the quantitative indicators of raw hides, such as weight, percentage of live weight, size, surface area in dm² and thickness in certain body parts in cull cows from Limousin, Galloway, Normande Cattle and Bulgarian Red Cattle breeds, which had completed their growth and were excluded from breeding for various reasons.

MATERIALS AND METHODS

The experiment was conducted with animals from the farm of Ivan Ivanov - an agricultural producer from the town of Aksakovo; animals from Kastelo Farm in the village of Slivovo Pole; and animals from the farm of the Experimental Base of RIMSA-Troyan in the late autumn of 2022 and the winter of 2023. The objective of the study was cows excluded from breeding for various reasons at the age of 5-10 years, which had completed their growth. The raw hides were studied of slaughtered cows from the following breeds: Limousin, Galloway, Normande cattle, and Bulgarian Red Cattle, at different ages and live weights, owned by the farms of agricultural producers, farmers, and the Research Institute of Mountain Stockbreeding and Agriculture. Five hides were studied from each breed. The cows were raised in identical conditions, in a free-stall housing system, and fed traditionally, a circumstance that contributed to the manifestation of their productive qualities. The slaughter and skinning took place in the Meat Factory of Svishtov, Meat Factory of Aksakovo, and Meat Factory of Slivovo Pole.

The weight and surface area of raw hides were determined after slaughter and cleaning using an electronic scale to the nearest of 0.01 kg. The sizes were measured by a retractable tape measure. The surface area of the hide was determined by the method of sum of squares in dm² by a measuring tape, and the thickness with a caliper at two specific points: at standard point ‘O’ in the hide of the withers and at standard point ‘H’ in the area of the sacrum according to the Arzumanyan method (1962).

The data were processed by the methods of variation statistics using the program Statistica-2010 and presented in tables and figures.

RESULTS AND DISCUSSIONS

The weight, percentage of live weight, size, surface area, and thickness of raw hides in specific sections of cull cows, which had completed their growth, are shown in Table 1.

Table 1. Technological indicators of raw hides of meat and combined (dairy-meat) cows, which had completed their growth, excluded from breeding for various reasons (X ± Sx)

Cattle Breed	n	Technological indicators of rawhide						
		Hide weight		Length, cm	Width, cm	Surface Area, dm²	Thickness at point ‘O’, mm	Thickness at point ‘H’, mm
		kg	% of live weight					
Limousin	5	41.37*±1.03	6.8	261.72±1.36	199.53±1.37	522.21±8.34	6.18**±0.26	6.68**±0.30
Galloway	5	39.46±1.24	7.0	234.01±4.23	189.56±2.47	443.57± 8.76	5.88±0.31	6.028*±0.25
Normande Cattle	5	47.23*±1.08	7.3	274.17±3.16	205.74*±1.09	564.70±9.54	7.12**±0.31	8.09**±0.21
Bulgarian Red Cattle	5	44.11*±1.11	7.2	249.97±1.19	198.79±1.32	496.91±10.81	6.47**±0.45	6.83**±0.35

*P<0.05, **P<0.01

The hides of two prospective beef cattle breeds, such as Limousin and Galloway, introduced in Bulgaria, and two combined breeds, such as Normande cattle and Bulgarian Red Cattle, were compared. The hides of different cattle breeds are distinguished by their structure and technological qualities. The weight of rawhide depends primarily on its size, thickness, sameness, and density (Kibkalo & Zhersbilov, 2009; Adzimova & Mambetov, 2018).

The size, thickness, and density of the hide have an impact on the realization of its potential. The steady increase in the weight of rawhide is not of the same character in different cattle breeds. The differences in the relative weight of the hide among the combined Normande cattle breed and meat breeds, such as Limousin and Galloway are clearly outlined, as it is 5.86 kg for the first breed and 7.77 kg for the second one, or 6.8% and 7.0% respectively.

There is a significant difference in the domestic combined breed Bulgarian Red Cattle with 3.12 kg or 7.2% respectively ($P<0.05$). In our opinion, the long-term use of Normande cattle in a combined productivity trend and the selected good meat, rounded forms, had an impact on the weight of its rawhide, which became higher, compared to previous studies.

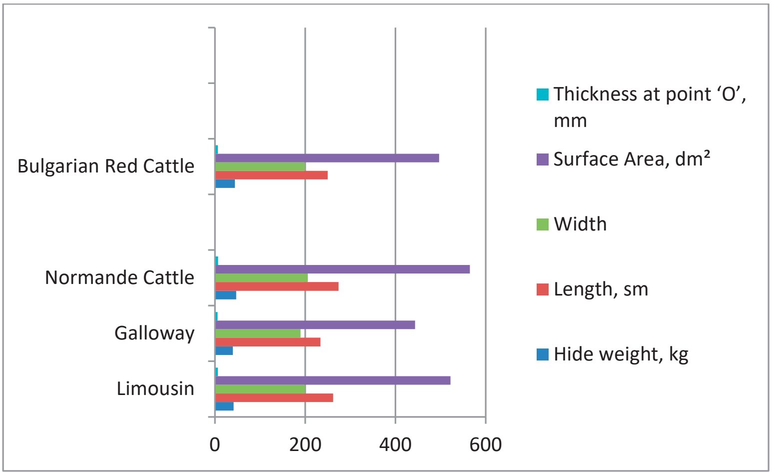

Figure 1. Graphical representation of technological indicators of raw hides from cows of meat and combined breeds, excluded from breeding for various reasons

The highest percentage of rawhide from the animal weight is registered for the Normande cattle breed with 7.3%, followed quite closely by the Bulgarian Red cattle breed with 7.2%, Galloway with 7.0% and Limousin with 6.9%. The value of hide in processing is determined by its surface area and thickness. The best represented in the space, in terms of spread, are the raw hides obtained from female animals of Normande cattle breed with 564.70 dm², followed by the Limousin breed with 522.21 dm², the hides of Bulgarian red cattle with 496.91 dm² and hides from Galloway breed with 443.57 dm², or differences of 42.49 dm², 67.79 dm² and 121.13 dm² ($P<0.05$). The differences among breeds on this indicator are not significant ($P<0.05$).

Table 2. The surface area of raw hides of different breeds in dm²

Cattle	Number (n = 5)	Area in dm^2	Area in dm^2 per 1 kg live weight
Normande cattle	5	564.70±3.44	0.903
Limousin	5	522.21±5.31	0.830
Bulgarian Red Cattle	5	496.91±12.81	0.831
Galloway	5	443.57±7.43	0.765

$P<0.05$

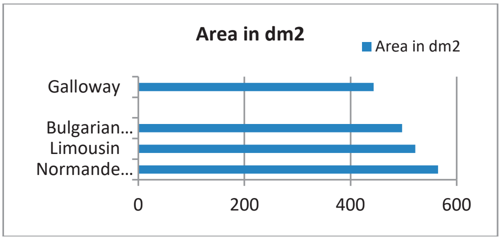

Figure 2. The surface area of raw hides of different breeds in dm²

The largest surface area of 564.70 dm² and 0.903 square decimeters per 1 kg of live weight was obtained from the representatives of Normande cattle breed, followed by Bulgarian Red cattle breed with 496.91 dm² and 0.831 square decimeters per 1 kg live weight, and Limousin with 522.21 dm² and 0.830 square decimeters per 1 kg live weight. The lowest results were obtained by the representatives of Galloway with 443.57 dm² and 0.765 square decimeters per 1 kg of live weight. The differences are explained by the rougher constitution and morphological structure of the Galloway breed.

The rawhide of adult animals is rougher and with uneven thickness in different sections. The thickest raw hides in the examined sections (point 'O' and point 'H') were measured in Normande cattle breed with 7.12 cm and 8.09 cm, followed by Bulgarian Red Cattle with 6.47 cm and 6.83 cm respectively, followed by Limousin breed with 6.18 cm and 6.68 cm respectively, whereas the thinnest hides were measured in the representatives of Galloway with 5.88 cm and 6.028 cm. The obtained results are significant at ($P<0.01$).

The data in the present study are close and correspond to the results of Sinivirski & Petkov, 1985; Kibkalo et al., 2014; Panin, 2015; Kozyr, 2018.

CONCLUSIONS

Cattle of different breeds and productivity trends, reared and fed under identical conditions, at approximately the same age and of the same sex, give raw hides different in relative weight and quality. The weight of the rawhide depends on the size, thickness, sameness, and density, and the quality depends on the spread, nutrition, and method of skinning.

Cattle breeds acclimatized in Bulgaria, both meat and dual-purpose, exhibit good qualitative and quantitative parameters of the resulting raw hides.

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ELECTROPHORETIC PATTERN OF SERUM PROTEINS IN CLINICALLY HEALTHY DAIRY COWS

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Abstract

For the diagnosis of diseases in cattle, apart from the clinical examination of the animal, the veterinarian also needs some practical, fast, cheap and, above all, available tools. The importance of this study lies in reducing the number of known paraclinical examinations that are often difficult to manage during a rural or farm field trip and are also expensive. Because clinical signs are often nonspecific, the general clinical examination of clinically healthy cows can only lead to a suspicion of disease without a necessary tool for confirmation or discovery of an insidious pathological process. For this reason, serum protein electrophoresis (SPE) is an important component of laboratory diagnostic evaluations for the measurement of serum proteins. In our study we used 8 clinically healthy cows of which 4 lactating cows and 4 weaned cows. Although there have been fluctuations in the value of various protein fractions and low specificities have been achieved in the diagnosis of certain diseases, the determination of the serum protein profile in ruminants and the correct interpretation of their results are very useful for clinicians in the clinical diagnosis of healthy and diseased animals and can serve as basis for other specific laboratory examinations, going up to protein fractions.

Key words*: albumins, biochemistry, blood serum, protein fractions, proteins.*

INTRODUCTION

Veterinary medicine has advanced a lot in recent years, but no matter how advanced the techniques available for diagnosis, in the evaluation of internal diseases of dairy cows we can never depart from the basic methods, starting from the complete clinical examination and exhaustive anamnesis, followed by the investigations common paraclinical tests such as hematological examination and microscopic evaluation of blood smear, serum biochemistry, urine examination, etc. In all animal species, gestation and lactation are characterized by intense metabolic changes and changes in many haematological and to chemical components, including variables related to protein metabolism (Bossuyt, 2006).

In clinical medicine, electrophoresis is mainly used to quantitatively separate and classify (electrophoretic pattern or pattern) the proteins in a biological sample (serum, urine, cerebrospinal fluid, peritoneal or pericardial fluid, etc.) and immunoelectrophoresis allows the qualita-tive detection of components to be named (Keren, 2003).

Electrophoresis is an analytical technique based on the migration of charged particles in a solution under the influence of an electric field (Thrall et al., 2012).

Protein fractions migrated by electrophoresis were identified in dairy cows, from cathode to anode, as albumin, alpha 1 - and alpha 2 - globulins, beta 1 - and beta 2 -globulins, and gamma globulins (Jackson et al., 2015; Ionita, 2024).

Factors regulating immunoglobulin production by normal and disease-associated plasma cells. Blood electrophoresis varies according to age, physiological state, body condition score, etc. The availability and speed of this investigation, its non-invasive nature, low cost and important diagnostic information justify the efforts made to obtain optimal results and a quality interpretation.

MATERIALS AND METHODS

Serum protein electrophoresis is a technique that consists in the separate migration of serum protein fractions on a semisolid gel, in the presence of an electric current.

The proteins are arranged on the gel in bands, according to their size and electrical charge, then the protein bands are colored with a special staining solution, so that they can be visualized. After the gel has dried, a densitometer measures the bands: the densitometer provides a visual pattern or graph of the protein bands, as well as a calculation of the relative percentage of each protein band in the total protein.
The only individual protein that is distinctly separated by electrophoresis is albumin, which has a clinical connotation only when it has low values compared to normal.
The biological material was represented by 8 collected blood samples, from which the serum was extracted and used for electrophoresis. The electrophoresis was carried out using the EP Line 1.0 electrophoresis system (Figure 1), consisting of an EP SA200 voltage source, an EP MB2 x 40 migration bath and a dedicated EP Line analysis, calculation and results interpretation system 1.0

Figure 1. The EP Line 1.0 electrophoresis system (original)

This system is adapted to a computer that allows data analysis and interpretation.

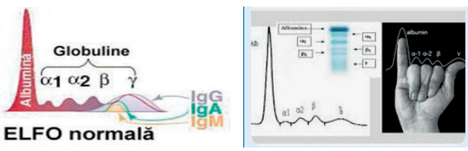

Figure 2. Normal electrophoresis (Bossuyt, 2006)

After the gel has dried, a densitometer measures the bands: the densitometer provides a visual pattern or graph of the protein bands, as well as a calculation of the relative percentage of each protein band in the total protein. The only individual protein that is distinctly separated by electrophoresis is albumin, which has clinical connotations only when it is significantly lower than normal (in fact albumin in pregnant cows is slightly lower). The electrophoretogram divides the protein fraction of serum or plasma into its constituent components, which include albumin and globulins. There are three globulin fractions: alpha, beta and gamma (Figures 3 and 4).

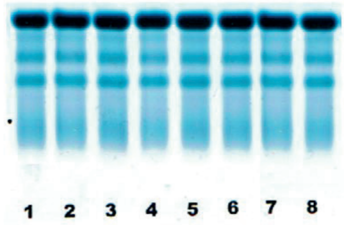

Figure 3. Samples with migrated and stained protein fractions (original)

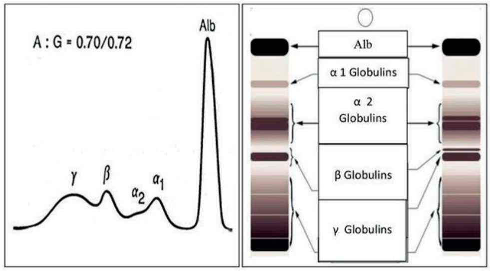

Figure 4. Example of healthy bovine serum protein electrophoresis (after Ionita L., 2024)

RESULTS AND DISCUSSIONS

The study was carried out in a farm of 50 cows from which we chose 8 dairy cows, of which 4 were lactating and 4 were weaned. The age of the monitored cows was between 3.5 and 6 years and they were in their second to fourth gestation. The cows were in good nutritional condition with an average body weight of about 550 kg. The health status of the animals during the evaluated period was observed daily. All animals were clinically healthy.

A. Dairy cows

Table 1. Blood biochemical examination of proteins

Lactating cows	Total protein g/dl	Albumin g/dl	Globulin g/dl	Report A/G	
1	**5.50**	**1.81**	3.69	0.49	
2		6.90	**3.82**	3.08	1.24
3		6.30	**4.32**	**1.98**	**2.19**
4		6.40	**4.39**	**2.01**	**2.19**
Mediate		6.28	3.59	**2.69**	**1.53**
Physiological values		5.8-8.5	2.5-3.7	3.3-4.8	0.45-1.31

From the analysis of Table 1, in dairy cows normal values of total serum proteins, but regarding albumin, 3 cows had increased albuminemia values (probably slightly dehydrated) and 1 sample was found below normal values (low during pregnancy).

Regarding the globulins, it is found that in samples 3 and 4 they are found to be low compared to normal. It should be noted that in samples 3 and 4 albumin was significantly increased, globulins significantly decreased, and the A/G ratio is above the physiological limit.

Regarding the average of the sample, it is found that the total proteins and albumin are within physiological limits, the globulins are low and the A/G ratio is increased above the physiological limit. This requires performing electrophoresis to see what happens to the protein fractions of these 4 samples.

Sample 1 - Dairy cow

Table 2. The results of the blood biochemical examination

Sample	Total protein g/dl	Albumin g/dl	Globulin g/dl	Report A/G
1.	**5.50**	**1.81**	3.69	0.49
Physiological values	5.8-8.5	2.5-3.7	3.3-4.8	0.45-1.31

In the blood biochemical examination (Table 2), total proteins and albumin are below normal values and globulins and the A/G ratio are within physiological values

Table 3. Electrophoresis of protein fractions

Parameter	Result g/dl)	Result (%)	Reference (%)
Alb	1.81	32.94	27-38
(H) Alpha 1	0.53	**9.7**	4-8
(H) Alpha 2	0.73	**13.32**	**4-8**
(H) Beta	1.18	**21.37**	**6-10**
(H) Gama	1.25	**22.67**	12-22

Analysis result:

Total protein = 5.50 g/dl, Report A/G = 0.49

From the Table 3 and Figure 5 of the electrophoretic examination in this animal, it is found that all globulin fractions are above normal values, albumin having normal values.

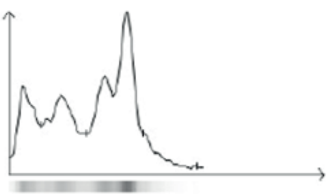

Figure 5. The graphic aspect of the migration of protein fractions

Sample 2 - Dairy cow

Table 4. The results of the blood biochemical examination

Sample	Total protein g/dl	Albumin g/dl	Globulin g/dl	Report A/G
2	6.90	**3.82**	**3.08**	1.24
Physiological values	5.8-8.5	2.5-3.7	3.3-4.8	0.45-1.31

From Table 4 in the blood bio-chemical examination total proteins and the A/G ratio were within physiological limits, albumin increased, and globulins decreased

Table 5. Electrophoresis of protein fractions

Parameter	Result (g/dl)	Result (%)	Reference (%)
Alb	3.82	**56.34**	**27-38**
(H) Alpha 1	0.38	5.44	4-8
(H) Alpha 2	0.57	**8.32**	**4-8**
(H) Beta	1.00	**14.43**	**6-10**
(H) Gamma	1.14	16.47	12-22

Analysis result:

Total protein = 6.90 g/dl, Report A/G = 1.24

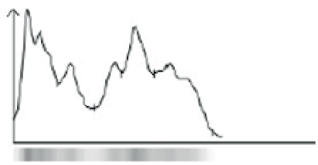

Figure 6. The graphic aspect of the migration of protein fractions

From the Table 5 and Figure 6 it follows that albumins and fractions Alpha 2 and beta were increased and fractions α1 and gamma globulins are within normal limits.

Sample 3 - Dairy cow

Table 6. The results of the blood biochemical examination

Sample	Total protein g/dl	Albumin g/dl	Globulin g/dl	Report A/G
3	6.30	**4.32**	**1.98**	**2.19**
Physiological values	5.8-8.5	2.5-3.7	3.3-4.8	0.45-1.31

Table 6 shows that total proproteins were within physiological limits, albumin was significantly increased, globulins were low, and the A/G ratio was very high.

Table 7. Electrophoresis of protein fractions

Parameter	Result (g/dl)	Result (%)	Reference (%)
Alb	4.32	**68.60**	**27-38**
(H) Alpha 1	9.31	4.98	4-8
(H) Alpha 2	0.54	**8.58**	**4-8**
(H) Beta	0.92	**14.57**	**6-10**
(H) Gamma	0.20	3.15	12-22

Analysis result:

Total protein = 6.30 g/dl, Report A/G = 2.19

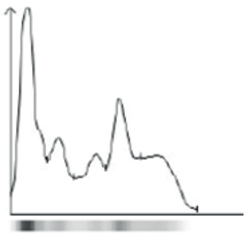

Figure 7. The graphic aspect of the migration of protein fractions

Table 6 and Figure 7 show that the α1 fraction was normal, the other globulins were increased, and the gamma fraction was decreased.

Sample 4 - Dairy cow

Table 8. The results of the blood biochemical examination

Sample	Total protein g/dl	Albumin g/dl	Globulin g/dl	Report A/G
4	6.40	**4.39**	**2.01**	**2.19**
Physiological values	5.8-8.5	2.5-3.7	3.3-4.8	0.45-1.31

Table 8 shows that total proteins were within physiological limits and albumins and the A/G ratio were very high and globulins very low.

Table 9. Electrophoresis of protein fractions

Parameter	Result (g/dl)	Result (%)	Reference (%)
Alb	4.39	68.63	27-38
(H) Alpha 1	0.34	6.30	4-8
(H) Alpha 2	0.36	5.64	4-8
(H) Beta	1.19	18.57	6-10
(H) Gamma	0.12	1.86	12-22

Analysis result:

Total protein = 6.40 g/dl, Report A/G = 2.19

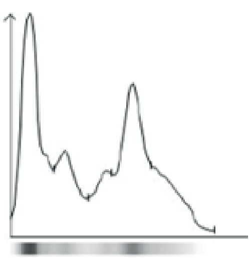

Figure 8. The graphic aspect of the migration of protein fractions

From the data obtained by electrophoresis, this sample shows an increase in albumin and β fractions and a very significant decrease in γ fractions (Table 9 and Figure 8).

B. The following results were observed in **weaned cows** in the period of mammary rest

Table 10. Blood biochemical examination of proteins

Lactating cows	Total protein g/dl	Albumin g/dl	Globulin g/dl	Report A/G
1	**5.50**	**1.81**	3.69	0.49
2	6.90	**3.82**	3.08	1.24
3	6.30	**4.32**	**1.98**	**2.19**
4	6.40	**4.39**	**2.01**	**2.19**
Mediate	6.28	**3.59**	**2.69**	**1.53**
Physiological values	5.8-8.5	2.5-3.7	3.3-4.8	0.45-1.31

From the Table 10 it follows that the average of the sample is within physiological limits in 3 samples, being only small fluctuations in a single sample (3) where the significant increase in albumin caused a significant decrease in globulins and the A/G ratio was well above the limit physiological.

Sample 1 - Weaned cow

Table 11. The results of the blood biochemical examination

Sample	Total protein g/dl	Albumin g/dl	Globulin g/dl	Report A/G
1	7.10	2.77	4.33	0.64
Physiological values	5.8-8.5	2.5-3.7	3.3-4.8	0.45-1.31

From Table 11 all the parameters of the analyzed protein profile are within normal limits.

Table 12. Electrophoresis of protein fractions

Parameter	Result (g/dl)	Result (%)	Reference (%)
Alb	2.77	**38.96**	**27-38**
(H) Alpha 1	0.41	5.79	4-8
(H) Alpha 2	0.60	**8.48**	**4-8**
(H) Beta	0.99	**13.88**	**6-10**
(H) Gamma	2.33	**32.88**	**12-22**

Analysis result:

Total protein = 7.10 g/dl, Report A/G = 0.64

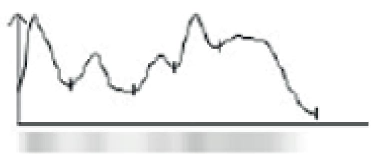

Figure 9. The graphic aspect of the migration of protein fractions

The electrophoresis results show that the α1 fraction was normal, the other globulins being slightly increased.

Sample 2 - Weaned cow

Table 13. The results of the blood biochemical examination

Sample	Total protein g/dl	Albumin g/dl	Globulin g/dl	Report A/G
2	6.40	3.03	3.37	0.90
Physiological values	5.8-8.5	2.5-3.7	3.3-4.8	0.45-1.31

From Table 13 all the parameters of the analyzed protein profile are within normal limits.

Table 14. Electrophoresis of protein fractions

Parameter	Result (g/dl)	Result (%)	Reference (%)
Alb	3.03	**47.28**	**27-38**
(H) Alpha 1	0.43	6.75	4-8
(H) Alpha 2	1.03	**16.04**	**4-8**
(H) Beta	1.17	**18.35**	**6-10**
(H) Gamma	0.74	**11.57**	**12-22**

Analysis result:
Total protein = 6.40 g/dl, Report A/G = 0.90

Figure 10. The graphic aspect of the migration of protein fractions

From the Table 14 and the Figure 10, we can see normal values of Alpha 1 globulins, albumins and β globulins are increased and gamma globulins are very slightly decreased compared to normal.

Sample 3 - Weaned cow

Table 15. The results of the blood biochemical examination

Sample	Total protein g/dl	Albumin g/dl	Globulin g/dl	Report A/G
3	6.90	**4.31**	**2.59**	**1.68**
Physiological values	5.8-8.5	2.5-3.7	3.3-4.8	0.45-1.31

From the Table 15 the total proteins are in physiological values, the albumin and the A/G ratio are significantly increased, and the globulins are at low values.

Table 16. Electrophoresis of protein fractions

Parameter	Result (g/dl)	Result (%)	Reference (%)
Alb	3.82	**56.34**	**27-38**
(H) Alpha 1	0.38	5.44	4-8
(H) Alpha 2	**0.67**	**8.32**	**4-8**
(H) Beta	**1.00**	**14.43**	**6-10**
(H) Gamma	1.14	16.47	12-22

Analysis result:
Total protein = 6.90 g/dl, Report A/G = 1.68

Figure 11. The graphic aspect of the migration of protein fractions

From Table 16 and Figure 11, alpha 1 albumins and gamma globulins are in physiological values and albumin and alpha 2 and beta fractions are increased.

Sample 4 - Weaned cow

Table 17. The results of the blood biochemical examination

Sample	Total protein g/dl	Albumin g/dl	Globulin g/dl	Report A/G
3	6.70	3.42	3.28	1.04
Physiological values	5.8-8.5	2.5-3.7	3.3-4.8	0.45-1.31

From Table 17 all the parameters of the analyzed protein profile are within normal limits.

Table 18. Electrophoresis of protein fractions

Parameter	Result (g/dl)	Result (%)	Reference (%)
Alb	3.42	**51.01**	**27-38**
(H) Alpha 1	0.37	5.54	4-8
(H) Alpha 2	0.55	**8.18**	**4-8**
(H) Beta	1.22	**18.20**	**6-10**
(H) Gamma	1.14	17.06	12-22

Analysis result:
Total protein = 6.70 g/dl, Report A/G = 1.04

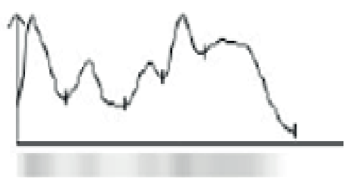

Figure 12. The graphic aspect of the migration of protein fractions

From Table 18 and Figure 12, alpha 1 albumins and gamma globulins are in physiological values and albumin and alpha 2 and beta fractions are increased.

CONCLUSIONS

1. Electrophoresis is a practical and economical alternative in the clinical exploration of ruminant medicine.
2. Electrophoresis is a non-invasive test that complements the quantitative characterization of proteinemia with information on fraction composition that can also be followed in evolution.
3. Even if by biochemical determinations the total proteins and albumins are in physiological values, by electrophoresis significant fluctuations of the protein fractions may appear which may betray an asymptomatic or chronic pathology in clinically healthy dairy cows.
4. Despite this low specificity in the diagnosis of certain diseases, the determination of the serum protein profile in ruminants and the correct interpretation of their results are very useful for clinicians in the diagnosis of healthy and diseased animals and can serve as a basis for other specific laboratory examinations.

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COMPARATIVE STUDY OF THE LEUCOCYTARE FORMULA AND OF SOME BLOOD BIOCHEMICAL PARAMETERS TO ROSS 308 AND COBB 500 MEAT HYBRIDS

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Abstract

In poultry farming, thanks to the development of technology, the broiler is a remarkable achievement in terms of genetics, nutrition and economic performance. The monitoring of the disease state is done from a clinical and paraclinical point of view only when the situation requires it. The determination of hematological parameters provides valuable information for the assessment of the health status of animals, but in current avian pathology these determinations are not widely used due to the lack of reference values for avian blood profiles, the very young economic age of broilers in conditions where feeding, watering, animal hygiene are regulated and monitored daily. In addition, the fact that erythrocytes in birds are nucleated and the hematology machines do not distinguish erythrocytes from leukocytes, counting them as nucleated cells giving errors in the counting, we, in practical field conditions, resorted to performing the leukocyte formula (easier to performed even in farms) and to the laboratory determination of some biochemical parameters to achieve a small difference between the 2 broiler hybrids during the growth period. From the experiment data we found that.

Key words: *albumins, biochemistry, blood serum, protein fractions, proteins.*

INTRODUCTION

The broiler chicken regardless of the hybrid it comes from (Ross 308 and/or Cobb 500) gives the image of a healthy chicken; their growth presenting multiple advantages, such as: the accessibility of meat consumption (it has no religious prohibitions), food convenience (always fresh meat can be delivered), a large number can be grown on small areas, the meat has nutritional and dietary value, etc. (Ionita, 2024).
Poultry health refers to: well-being, providing biological comfort, ensuring balanced nutrition (quantitative and qualitative), etc., all of which will allow to achieve the genetic performance of these hybrid breeds Ross 308 and Cobb 500 (Talebi et al., 2005).
Modern commercial broiler hybrids are not all the same: they are very similar but have distinct growth characteristics.
Some produce larger breasts, others larger thighs and legs, while some produce balanced breast and leg/thigh meat.
Several operators focus on rapid growth and meat gain from hatch, while others focus on slower growth with an emphasis on structural development - limb bones and heart muscle (Broiler Management Guide) These growth traits are important to traders looking to produce meat for their specific market targets. With all the listed advantages, raising broilers requires the identification and application of appropriate biosecurity measures to prevent possible diseases with serious economic implications (Figures 1 and 2).

Figure 1. Advanced broiler breeding technology (www.aviagen.com)

Figure 2. The main risk factors of bird diseases (FAO, 2011)

Thanks to the immunoprophylactic measures applied for a very long time, no disease situations were recorded during the short economic period of 28-35 days when these chickens are raised. That's why hematological analyzes are not used either, and blood biochemical determinations are made only for scientific and economic purposes to improve the quality of the meat and influence it through the addition of nutrients to improve or correct certain targeted properties.

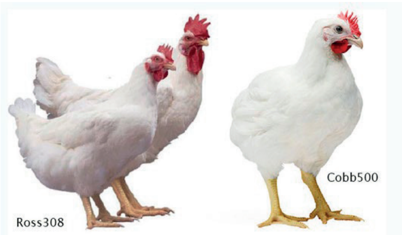

Figure 3. Broiler breeds Ross 308 and Cobb 500 (www.aviagen.com)

MATERIALS AND METHODS

To perform the metabolic Mini profile, we collected blood samples from broilers of different breeds, similar ages, 2 different rearing units, but from clinically healthy birds.
The collection was carried out in special vacutainers for biochemistry and hematology, by puncturing the humeral vein; for serum expression, the blood samples were kept at room temperature, then in the refrigerator at 4^0C.
The analyzes were performed in the Laboratory of the discipline of Internal Medicine, within FMV Bucharest, with the equipment provided.
The leukocyte formula was obtained through laboratory analysis using the hematology analyzer Abacus Junior Vet 5. It should be noted that this device could not be used for the quantitative determination of the red blood cell and its derivatives as well as the leukocyte blood cell because erythrocytes are nucleated (Figure 4).

Figure 4. Abacus Junior Vet 5 Hematology Analyzer (own source)

For biochemical blood analyses, serum samples were separated by centrifugation and measured by standard methods using the ARKAY apparatus (Figure 5).

Figure 5. ARKRAY Dry Biochemistry Analyzer, Model SPOTCHEM EZ-SP-4430 (own source)

To obtain the blood serum, the blood samples collected on the anticoagulant were centrifuged (Figure 6).

Figure 6. Centrifuge (own source)

To determine the leukocyte formula, we resorted to the hematology analyzer, which evaluated the percentage of the components of the white series, and I also made an image of them, from which nucleated erythrocytes and some white elements (heterophile, eosinophil, monocyte, lymphocyte) can be observed in Figure 7.

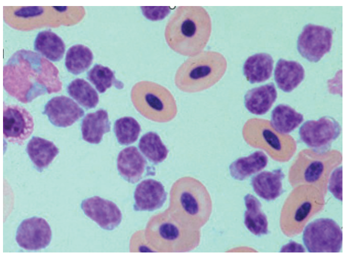

Figure 7. Microscopic field with red and white blood elements (own source)

RESULTS AND DISCUSSIONS

Currently in Romania but also in the European Union, the share in the growth of meat hybrids belongs to the Ross 308 hybrid (it has a fast development, efficiently utilizes the feed, etc.).
Ross 308 is a high-performance broiler that can reach impressive body weights (females around 3 kg and males 3.5 kg at 47 days of age); it has a very good yield at slaughter (Sam et al., 2023).
The Cobb 500 hybrid has a low feed conversion rate, fast growth, a less expensive nutritional regimen; has adaptation problems with an increased sensitivity to growth on permanent litter; it was oriented especially to produce *"breast"* (Talebi et al., 2005).

A. Ross 308 meat hybrids

Genetic heritage: Ross 308 is bred by Aviagen, a global poultry company. It is a cross between two different chicken breeds, each selected for specific traits such as growth rate, feed efficiency and meat quality.
It is globally recognized as a broiler that delivers consistent performance.
Integrated and independent producers appreciate its growth rate, feed efficiency and robust performance. Ross 308 boasts unmatched genetics optimized for fast growth, exceptional meat quality and efficient feed conversion.
Its adaptability to various environments and robust health profile makes it a reliable option for both small-scale and commercial operations.
This breed dominates the market, trusted by poultry producers for its proven track record and superior attributes.
The Ross 308 hybrid (Figure 8) requires a differentiated feed to achieve high performance but shows better resistance to microclimate conditions.

Figure 8. Ross 308 hybrid at 21 days of age (own source)

In Romania, it is grown on a large scale because the parents of Ross meat hybrids have a higher percentage of eggs compared to the parents of Cobb and because the hatching percentage is higher.
Especially in birds, the clinical diagnosis is completed with the laboratory examination, based on the results of hematological and biochemical investigations (Al-Aufi et al., 2024).

Table 1. Average biochemical values in 28-day-old Ross 308 chickens (n = 20)

Parameters	UM	Physiological values	Age 28 days (mediate)
Total proteins	g/dl	3-7	3.5
Albumins	g/dl	2.6-4.6	**1.42**
Glbobulins	g/dl	1.4-2.4	2.1
Uric acid	mg/dl	1.6-4.2	**17.3**
Triglycerides	mg/dl	25-500	145.0
Cholesterol	mg/dl	50-400	225.0
GOT	UI/l	125-500	**505.2**
GPT	UI/l	26-34	**38.9**
Bilirubin	mg/dl	0.2-4.0	1.2
Creatinine	mg/dl	0.3-0.9	0.4

Table 1 shows that uric acid is slightly increased, albumin is decreased and GOT and GPT are increased.

Table 2. Average biochemical values in 38-day-old Ross 308 chickens (n = 20)

Parameters	UM	Physiological values	Age 38 days (mediate)
Total proteins	g/dl	3-7	3.4
Albumins	g/dl	2.6-4.6	**2.3**
Glbobulins	g/dl	1.4-2.4	1.1
Uric acid	mg/dl	1.6-4.2	**26.7**
Triglycerides	mg/dl	25-500	125.5
Cholesterol	mg/dl	50-400	268.0
GOT	UI/l	125-500	432.0
GPT	UI/l	26-34	**42.0**
Bilirubin	mg/dl	0.2-4.0	1.6
Creatinine	mg/dl	0.3-0.9	0.7

Table 2 shows small variations in the biochemical parameters compared to broilers from the age of 28 days.
Albumin is the most important plasma protein substance that is formed in the liver; permanent exchanges between serum albumins and tissues explain the frequency of quantitative albumin variations in various pathological states. The decrease in albumin can be due to the decrease in liver production, infectious processes, acute inflammation, protein losses at the renal, gastrointestinal level etc.

A proteinemia of more than 3 mg/dl may be associated with albumin loss through abnormal fluid distributions in the body, such as abdominal ascites or effusions around organs; these two aspects can be encountered at slaughter without being diagnosed by laboratory examination. In birds, uric acid is the main molecule of nitrogen catabolism and plays a vital role; there is a direct relationship between the amount of protein ingested and the level of uric acid in the blood.

The high concentration of protein in the forage ration during the growing period causes an increase in the level of uric acid in the blood.

The appearance of the leukocyte formula in the Ross 308 hybrid falls within the physiological limits at both 28 and 38 days of age.

Table 3. Leukocyte formula in Ross 308 hybrids, at the age of 28 and 38 days (n = 20)

Parameter	U/M	Physiological values	Mediate chicken 28 days	Mediate chicken 38 days
Lymphocytes	%	57-93	60.1	63.7
Heterophiles	%	8-48	34.5	22.9
Eosinophils	%	1-8	2.6	**11.6**
Basophils	%	0-3	0.8	0.6
Monocytes	%^	0-7	2	1.2

Table 3 shows eosinophilia in 38-day-old chicks (at finishing).

In the routine practice of clinical laboratories, it is possible to determine both the proportion of eosinophils per 100 leukocytes counted in the pan optically stained blood smear and the number of eosinophils per mm of peripheral blood allows their identification even when the leukocyte formula leads to false positive results due to the uneven distribution of blood cells on the smear. In recent years, the techniques for identifying and counting blood elements have been automated, significantly simplifying the analysis and increasing the accuracy of the results.

Next, we present the hematological and blood biochemical situation of the Cobb 500 hybrids

B. Cobb 500 meat hybrids

Selected for breeder and broiler genetic excellence, Cobb 500 offers unmatched durability and uniformity, exceptional feed efficiency and outstanding growth rate. Today's modern broiler chickens are more efficient, productive and robust than previous generations. This progress is due to Cobb's commitment to improving genetics and advances in breeding methods that increase performance potential and enhance longevity and welfare outcomes. A cross-characteristic COBB 500 broilers - white feathers and genetically yellow skin, so that even when fed normal diets the unpigmented skin of the finished carcass is always yellow. Compared to other broiler crosses, COBB 500 has higher productivity and less growth in the fattening period. In 35 days, the average weight is 1.9 kg, it should be in 42 days - 2.4 kg (Figure 9).

Figure 9. The Cobb 500 hybrid at the finish (own source)

Table 4. Average biochemical values in 28-day-old Cobb 500 chickens (n = 20)

Parameters	UM	Physiological values	Age 28 days (mediate)
Total proteins	g/dl	3-7	3.16
Albumins	g/dl	2.6-4.6	**1.18**
Glbobulins	g/dl	1.4-2.4	1.98
Uric acid	mg/dl	1.6-4.2	**8.15**
Triglycerides	mg/dl	25-500	97.5
Cholesterol	mg/dl	50-400	143.3
GOT	UI/l	125-500	218.3
GPT	UI/l	26-34	**18.0**
Bilirubin	mg/dl	0.2-4.0	0.30
Creatinine	mg/dl	0.3-0.9	**1.91**

Table 4 shows the increase in uric acid and creatinine and the slight decrease in albumin.

Table 5. Average biochemical values in 38-day-old Cobb 500 chickens (n = 20)

Parameters	UM	Physiological values	Age 38 days (mediate)
Total proteins	g/dl	3-7	2.88
Albumins	g/dl	2.6-4.6	**1.09**
Glbobulins	g/dl	1.4-2.4	1.79
Uric acid	mg/dl	1.6-4.2	**5.48**
Triglycerides	mg/dl	25-500	46.1
Cholesterol	mg/dl	50-400	138.9
GOT	UI/l	125-500	276.9
GPT	UI/l	26-34	28.7
Bilirubin	mg/dl	0.2-4.0	0.64
Creatinine	mg/dl	0.3-0.9	**1.36**

Table 5 shows that in broilers at the age of 38 days, at the finishing stage, uric acid and creatinine were found to be slightly increased and the albumin is low

The appearance of the leukocyte formula in the Cobb 500 hybrid falls within the physiological limits at both 28 and 38 days of age.

Table 6. Leukocyte formula in Cobb 500 hybrids, at the age of 28 and 38days (n = 20)

Parameter	U/M	Physiological values	Mediate chicken 28 days	Mediate chicken 38 days
Lymphocytes	%	57-93	63	67
Heterophiles	%	8-48	32	26
Eosinophils	%	1-8	3	4
Basophils	%	0-3	1	1
Monocytes	%^	0-7	1	2

Regarding the leukocyte formula, in Cobb 500 hybrids it is found that all types of white cells (granulocytes and agranulocytes) are within physiological limits (Table 6).

Figure 10. Cobb 500 broiler house (www.aviagen.com)

Next, we present some comparative metabolic results between the 2 hybrid broilers.

Table 7. Comparative protein profile

Parameters	Ross 308 (mediate)		Cobb 500 (mediate)	
	28 days	38 days	28 days	38 days
Total proteins	3.5	3.4	3.16	2.88
Albumins	1.42	2.3	**1.18**	**1.09**
Glbobulins	2.1	**1.1**	1.98	1.79

The analysis of the data shows (Table 7) that in the hybrid Cobb 500 albumin is found below the physiological value at both investigated ages (28 and 38 days).

Table 8. Comparative energy profile

Parameters	Ross 308 (mediate)		Cobb 500 (mediate)	
	28 days	38 days	28 days	38 days
Triglycerides	145.0	125.5	97.5	46.1
Cholesterol	225.0	268.0	143.3	138.9

From the analysis of the results (Table 8), it is observed that the parameters of the energy profile, in both hybrids, are within physiological limits.

Table 9. Comparative enzyme activity between broilers

Parameters	Ross 308 (mediate)		Cobb 500 (mediate)	
	28 days	38 days	28 days	38 days
GOT	**505.2**	432.0	218.3	276.9
GPT	**38.9**	**42.0**	**18.0**	28.7

Regarding liver activity (intensely forced by growth), a significant increase in GOT and GPT activity is recorded in hybrid Ross 308, at both tested ages (Table 9). A very good activity of these transaminases is found in Cobb 500 hybrids.

Table 10. Comparative activity of uric acid

Parameters	Ross 308 (mediate)		Cobb 500 (mediate)	
	28 days	38 days	28 days	38 days
Uric acid	**17.3**	**26.7**	**8.15**	**5.48**

Birds are known to be hyperuricemic normally. Of the 2 hybrids, it appears that Cobb 500 have uric acid values close to physiological values, including during the low finishing period. Ross 308 hybrids have high uric acid values (Table 10). This increase in uricemia is related to the period of intensive growth of the chicks, a stage of intense metabolic demand.

CONCLUSIONS

1. Modern commercial broiler hybrids are not all the same - they are very similar but have distinct growth and metabolism characteristics.
2. Growth traits are important to commercial agents who wish to produce meat for their specific market objectives.
3. Hematological, the laboratory analyzes revealed that the leukocyte formula is within normal limits, only in Ross 308 hybrids at the age of 38 days a marked eosinophilia was found
4. From a biochemical point of view, comparatively, the results show a significant increase in GOT and GPT activity in the Ross 308 hybrid, at both investigated ages

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EFFECT OF DIFFERENT HORMONAL PROTOCOLS WITH TIMED ARTIFICIAL INSEMINATION ON CLINICAL SIGNS OF ESTRUS AND CONCEPTION RATES IN BULGARIAN MURRAH BUFFALOES

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Abstract

In buffaloes, synchronization is especially important because of the peculiarities of reproduction. Gestation was diagnosed sonographically 45 days after timed AI (TAI) in 133 buffalo cows and 75 heifers hormonally treated. The dispersion analyses included the factors-classes: protocol - PreSynch/OvSynch, OvSynch, OvSynch+PRID; clinical signs of estrus (CSE) - without CSE, sole patency of cervix, mucous discharge; age – heifers, cows; season - in and out of season. The results show that protocol has strongest effect on conception rates (CR) ($P \leq 0.01$), significantly lowest being that under PreSynch/OvSynch - 23.9%, compared to OvSynch (40.7%) and OvSynch+PRID (47.4%). In the heifers OvSynch+PRID and OvSynch show markedly higher results (50.0 and 52.9%), while in the buffalo cows with OvSynch CR is relatively low (38.3%). CSE is also significant ($P \leq 0.05$), the highest CR in the cases with mucus. The superiority of OvSynch+PRID finds expression in the highest incidence of full estrus (mucus) in the lactating buffaloes (70%) and especially in the heifers (82%). Especially OvSynch+PRID can be used for overcoming seasonal anestrus.

Key words: *buffaloes, conception rate, estrus signs, PRID, prostaglandin.*

INTRODUCTION

Management of herd replacement and profitability in buffalo farming are majorly based on age of first calving and calving interval, rather than on milk yield, which is proved with the economic weights of the traits in the Bulgarian Murrah (Peeva, 2000) and in the Italian Mediterranean breed (Barile, 2005). In particular, breeding efficiency of buffaloes is affected by late puberty, seasonality of calving, prolonged anestrus after calving, irregular cyclycity, low conception rates, etc. (Madan 1990). The great variability of estrus and ovulation is a challenge to AI, as well (Ohashi, 1994; Perera, 2011).

Moreover, the reproductive process in the buffalo farms is specifically characterized by vaguely expressed signs of estrus and hence by poor timing and low success of artificial insemination (Alonso et al., 1992; Barkawi et al., 1993), and in addition the portion of animals in long anestrus is great, especially in the non-breeding season (El-Wishy, 2007). It is a characteristic of the species that high sexual activity comes in response to the reducing day light in late summer and early autumn in association with melatonin secretion, while in the rest of the year buffaloes often exhibit anestrus which, on one hand, reduces reproductive efficiency and, on the other, results in a misbalanced milk production throughout the year (Zicarelli, 1997; 2007; Neglia et al., 2004).

Efficiency of protocols for estrus and ovulation synchronization has been established to increase the percentage of pregnant animals (Baruselli & Carvalho, 2005; Balamurugan et al., 2017). Further, combined administration of gonadotropins and prostaglandins has been introduced with the purpose to optimize the effect of these protocols by the inclusion of timed artificial insemination (De Rensis & López-Gatius, 2007; Hammam et al., 2009). Consequently, certain success has been achieved in eliminating the effect of breeding season (Carvalho et al., 2013; Baruselli et al., 2013).

In buffalo breeding, the applied synchronization schemes (Paul & Prakash, 2005; Carvalho et al., 2007; Warriach et al., 2008) are of even greater importance, compared to cattle, in view of the above mentioned species-specific peculiarities of reproduction. Namely, the new follicular wave they induce affords circumventing the species-problematic visual heat detection for AI timing (Barile, 2005). There are only few research works studying the presence of clinical signs of full estrus in synchronized buffaloes on global (Neglia et al., 2003) and national (Atanasov et al., 2012) scale, suggesting that mucous discharge can be used as an indicator for good conception rates.
The objective of the presented field experiment was to study the efficacy of the application of three different protocols for estrus synchronization in buffalo heifers and buffalo cows of the Bulgarian Murrah breed expressed in manifestation of clinical signs of estrus and conception rates.

MATERIALS AND METHODS

The field experiment was carried out in the years 2021 and 2022 and included 75 buffalo heifers and 133 buffalo cows of the Bulgarian Murrah breed bred on the farm of Agricultural Institute – Shumen. The two categories of buffaloes are kept in tie-stall housing with exercise yards.
The diet of the buffalo cows includes 22 kg of maize silage, 3 kg of leguminous hay, 4 kg of cereal straw and 5 kg of compound feed per capita per day. The concentrate feed provides 1629 kcal of energy and 96 g of digestible protein and has the following composition: wheat - 15%, barley - 12%, corn - 56%, wheat bran - 10%, sunflower cake - 5%, dicalcium phosphate 0.6%, salt - 0.4% and chalk - 1%. The diet of the heifers consists of 18 kg of silage, 3 kg of hay and 4 kg of feed.
The buffaloes were selected to be up to sixth lactation and more than 60 days *postpartum*, and the heifers - to be roughly 22 months old with a live weight of about 380 kg. All included animals were in good body condition (BCS= 3-4) and free of obstetrical disorders and abnormalities in their prior parities (if any). On day 0, ovarian structures were examined sonographically and for treatment were chosen individuals with diameter of the follicles over 8 mm and no *corpus luteum*.
Three protocols of hormonal treatment for estrus induction/synchronization with timed artificial insemination (TAI) were applied in heifers and lactating buffaloes, as presented in Table 1. In the lactating buffalo cows, each of the protocols was applied both in breeding and non-breeding season, while in the heifers - out of season only. Gestation was also diagnosed sonographically on the 45th day after TAI. The echographic examinations were performed with a SonoScape A2 Vet (SonoScape Co. LTD, Shenzhen, China), a multi-frequency (7-12 MHz) linear probe and transrectal approach.

Table 1. Timing of hormonal administration within the three tested protocols

Date/Hour		PreSynch/OvSynch (n_H= 46; n_c= 46)	OvSynch (n_H= 12; n_c= 47)	OvSynch+PRID (n_H= 17; n_c= 40)
Day 1	8 a.m.	500 UI Synchrostim[1] i.m. + 5 ml Enzaprost T[2] i.m.	2 ml Ovarelin i.m.	PRID DELTA[4] vaginally inserted
Day 4	8 a.m.	2 ml Ovarelin[3] i.m.		
Day 8	8 a.m.		5 ml Enzaprost i.m.	PRID DELTA removal + 5 ml Enzaprost i.m.
Day 10	4 p.m.		2 ml Ovarelin i.m.	2 ml Ovarelin i.m.
Day 11	8 a.m.	5 ml Enzaprost T i.m.	TAI	TAI
	4 p.m.			TAI
Day 13	4 p.m.	2 ml Ovarelin i.m.		
Day 14	8 a.m.	TAI		

i.m. – intramuscular injection; n_H – number of buffalo heifers, n_c – number of buffalo cows;
[1]Equine Serum Gonadotropin, *Ceva Sante Animale, France*; [2]Dinoprost Trometamol, *Ceva Sante Animale, France*; [3]Gonadorelin, *Ceva Sante Animale, France;* [4]Intrauterine device for cattle PRID delta 1.55 g Progesterone, *Ceva Sante Animale, France*

The criteria for the diagnosis of pregnancy were an enlarged uterine lumen, visualization of the embryonic vesicle, and foetal cardiac activity, as described by Fricke et al. (2016).
The artificial insemination and evaluation of the clinical signs of the genital apparatus associated with estrus were performed by an AI technician. The presence/absence of a clear cervical-vaginal mucous discharge was detected, as well as the passability of the cervical canal with a pipette (patency). Cryopreserved semen from tested buffalo bulls was used.
Examinations and treatments were carried out in compliance with the requirements and regulations of the Animal Welfare Act (AWA).
The set of data registered sonographically were processed via dispersion analysis of a non-orthogonal set of qualitative traits (Model-1), including the following factors and respective classes:
- Age – two classes: heifers; cows;
- Protocol – three classes: PreSynch/OvSynch; OvSynch; OvSynch+PRID;
- Clinical signs of estrus (CSE) as detected during TAI application – three classes: without CSE (lack of vaginal mucous discharge and low patency of the cervix); patency (good patency but lack of mucus); mucus (both presence of mucous discharge and good patency, i.e. expression of full estrus).

Another 3-factor analysis was conducted on buffalo cows only. Instead of age, Model-2 included the factor: season - two classes: in season (high breeding season); out of season (low breeding season). In-season TAI breeding was applied in the period from August 10th to November 21st. The rest of the year was considered out of season.
In the linear model, the gradations are presented as p_i values, resulting from the number of the individuals characterized by the qualitative trait pregnancy as diagnosed 45 days post TAI (Σm_x) out of the total number of individuals in the respective class (Σn_x). The effects of the singular factors included in the ANOVA, their co-effects and the all-factors effect (x) are expressed in coefficients of impact (η^2) and coefficients of significance (F), and the significance of the differences within gradations – in F_d-values.

RESULTS AND DISCUSSIONS

Table 2 shows the raw data from the ultrasound examinations for the diagnosis of pregnancy 45 days after TAI, as well as the visual detection of the presence of vaginal mucus and the patency of the cervical canal during TAI. It is noteworthy that in TAI after application of the PreSynch/OvSynch protocol, 17.4% of the heifers have shown no visual CSE, but still one animal conceived (12.5%).
This indicates that this protocol had low success in the heifers, while under the OvSynch and OvSynch+PRID scheme all young animals manifested at least patency of cervical canal.

Table 2. Results from day 45 post TAI by protocols and by CSE and distribution by high or low season of breeding

Protocols	CSE	n_x	m_x	Season, n_x/m_x	
				High	Low
Buffalo heifers					
PreSynch/ OvSynch	No CSE	8	1	-	46/8
	Patency	14	2		
	Mucus	24	5		
OvSynch	No CSE	0	0	-	12/6
	Patency	6	4		
	Mucus	6	2		
OvSynch+ PRID	No CSE	0	0	-	17/9
	Patency	3	1		
	Mucus	14	8		
Buffalo cows					
PreSynch/ OvSynch	No CSE	1	0	38/11	8/3
	Patency	19	4		
	Mucus	26	10		
OvSynch	No CSE	3	1	25/9	22/9
	Patency	20	5		
	Mucus	24	12		
OvSynch+ PRID	No CSE	5	1	16/6	24/12
	Patency	7	2		
	Mucus	28	15		

Accordingly, as indicated by the data in Figure 1, the pregnancy rate from the PreSynch/OvSynch protocol in the heifers was markedly lower as compared to the OvSynch and OvSynch+PRID synchronization schemes.
In the buffalo cows under the PreSynch/OvSynch protocol, conception rate was also lowest, but with less pronounced differences with OvSynch and OvSynch+PRID (Figure 1). It is noteworthy that while in the heifers OvSynch+PRID and OvSynch lead to high results (conception rates of 50.0 and

52.9% respectively), in the buffalo cows OvSynch has lower success (38.3%).

With no CSE in the PreSynch/OvSynch protocol was only one buffalo cow (2.2%) and she did not conceive; in each of OvSynch and OvSynch+PRID scheme there was one animal, which constitutes respectively 6.4 and 12.5%, with pregnancy rates of 33.3 and 20.0 % (Table 2).

It is noteworthy that in OvSynch the results when patency is the only CSE detected are even lower, while when mucous discharge is present the conception rate is markedly higher.

The differences in the manifestation of full estrus are remarkable. Figure 1 indicates that the heifers with mucus constitute as much as 82.4% of all OvSynch+PRID treated animals, compared to 52.2 and 50.0% in the other protocols. Similar but slightly less-expressed dependency is observed in the buffalo cows – 70.0% in OvSynch+PRID, 56.5% in PreSynch/OvSynch and 51.1% in OvSynch.

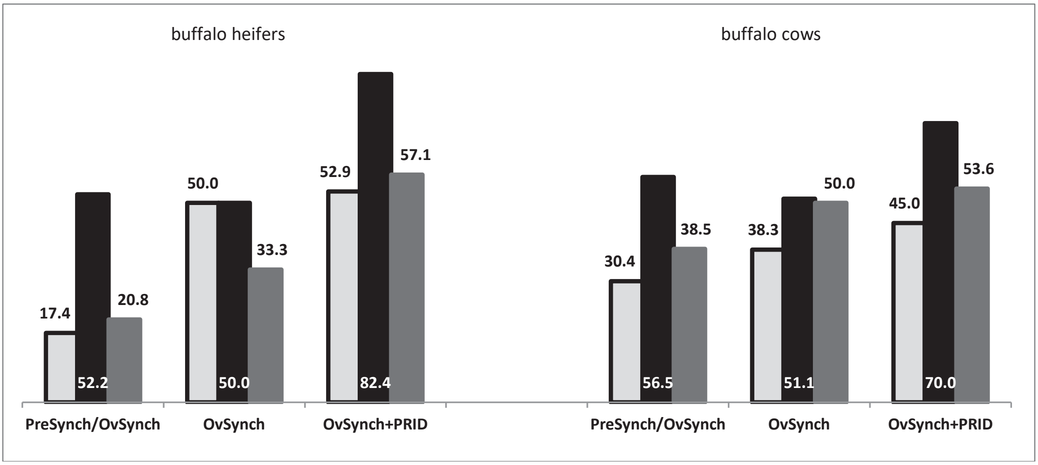

Figure1. Results based on raw data about overall conception rates (framed grey), incidence of full estrus (black) and conception rates from full estrus (dark grey) per protocol within age group, %

Obviously, as Table 2 shows, the PreSynch/OvSynch protocol results in relatively good percentage of heifers with mucous discharge but also in poor follicular wave associated with poor conception rates (Figure 1).

Figure 1 also shows that in both age groups the conception rates from full estrus are highest after application of TAI within the OvSynch+PRID protocol (over 50%), but in the lactating buffaloes OvSynch also has a high result (50.0%).

It is noteworthy that in the OvSynch scheme the heifers show good conception rates when their cervix has good patency and no mucus. Pregnancies in the buffalo heifers under the OvSynch+PRID are achieved majorly in the presence of mucous discharge (i.e., of both clinical signs), which applies also to the buffalo cows treated under the all three protocols.

ANOVA from the dispersion analysis in Model-1 indicates that the factor protocol has the most pronounced effect on conception rates – P≤ 0.01 (Table 3). The significantly lowest p_i-estimate belongs to the animals treated with PreSynch/OvSynch (Table 4), which is commensurate with the information in Figure 1 within age groups. CSE also has significant effect on the success of TAI (P≤ 0.05, Table 3), the highest p_i-value observed in the cases with mucous discharge during insemination.

Table 3. ANOVA from the dispersion analysis of conception rates, whole dataset - Model-1

Sources of variance	df	η^2	F	P
Age	1	0.0049	1.027	P> 0.05
Protocol	2	0.0463	4.894	**P≤ 0.01**
CSE	2	0.0373	3.948	**P≤ 0.05**
Age × Protocol	2	0.0505	5.340	**P≤ 0.01**
CSE × Protocol	4	0.0180	0.953	P> 0.05
CSE × Age	2	0.0595	6.287	**P≤ 0.001**
x	17	0.1016	1.265	P> 0.05
z	190	0.8984		
y	207	1.0000		

Although Figure 1 suggested some differences between heifers and cows, the dependence of conception rates on protocol and CSE have similar trends in the two categories of buffaloes. In this context are the data in the ANOVA (Table 3), showing that age is not a significant source of variance of pregnancy rates.

Table 4. Effect of the significant factors from ANOVA on conception rates, whole dataset – Model-1

Classes	Σn_x	Σm_x	p_i
Protocol			
1. PreSynch/OvSynch	92	22	0.239
2. OvSynch	59	24	0.407
3. OvSynch+PRID	57	27	0.474
Clinical signs of estrus (CSE)			
4. Without CSE	17	3	0.177
5. Patency	69	18	0.261
6. Mucus	122	52	0.426
Σ /Средно	208	73	0.351

Σn_x – number of individuals in the class/gradation
Σm_x – number of individuals echographically diagnosed pregnant on day 45 post TAI out of Σn_x
Significance of differences among p_i values within gradation: 1-[2, 3]* and 6-[4, 5] at $P \leq 0.05$

On the other hand, the co-effects of age with the factors protocol ($P \leq 0.01$) and CSE ($P \leq 0.001$) are significant. The co-effect of protocol with CSE and the all-factors co-effect, expressed as *x*-value, are not significant.

The ANOVA under Model-2 (Table 5) shows that the factor protocol is not a significant source of variation of conception rates in the lactating buffaloes, which corresponds with the above observed smaller differences in conception rates between high and low breeding season in the buffalo cows than in the heifers (Figure 1).

Table 5. ANOVA from the dispersion analysis of conception rates in lactating buffaloes - Model-2

Sources of variance	df	η^2	F	P
Season	1	0.0137	1.794	P> 0.05
Protocol	2	0.0147	0.962	P> 0.05
CSE	2	0.0586	3.855	**P≤ 0.05**
Season×Protocol	2	0.0953	6.256	**P≤ 0.001**
CSE×Protocol	4	0.0504	1.653	P> 0.05
CSE×Season	2	0.0514	3.371	**P≤ 0.05**
x	17	0.1237	0.955	P> 0.05
z	115	0.8763		
y	132	1.0000		

This implies that the above established differences among protocols depend mostly on those in the heifers. More importantly, the analysis of variance indicates that season does not affect pregnancy rates significantly, as well. Table 2 also shows the timing of the protocols as per season. It is noteworthy that the conception rate after out-of-season TAI under the OvSynch+PRID protocol is higher (50.0%) than in the main breeding season (37.5%). The differences between the seasons in the protocols PreSynch/OvSynch and OvSynch are smaller, implying a tendency to diminish the impact of season on buffalo reproduction.

On the basis of the observation in the presented field trial that high conception rates are achieved in the cases with both clinical signs (full estrus) only, we share the opinion in other works with the OvSynch protocol in the Bulgarian Murrah (Atanasov et al., 2012) that the presence of cervical mucus during AI can be used as an indicator for good conception rates, all the more that in our study the incidence of this clinical sign is much higher. Moreover, this rationale applies also to the OvSynch+PRID protocol where the proportion of buffalo cows and heifers with induced full estrus is definitely highest.

As in the breeds Murrah (Ghumen et al., 2014), Surti (Patel et al., 2022), Jafarabadi (Raval et al., 2021) and Italian Mediterranean (Presicce et al., 2004; De Rensis et al., 2005), field experiments on buffalo heifers and cows have resulted in very good estrus-inducing effect of the OvSynch protocol (under the same GnRH-$PGF_{2\alpha}$-GnRH scheme), an additive favorable effect on conception rates afforded by progesterone treatment in anestrus in high and low breeding season.

Applying out-of-season treatment with PreSynch/OvSynch, in buffalo heifers was achieved high estrus induction and conception rate which were close to the results from the other two protocols (Sing et al., 2010). However, according to these and to other Indian authors (Saini et al., 1988; Andurkar et al., 1995; Kumar et al., 2010), the sole application of this protocol does not provide good outcome, while in combination with progesterone inserts or prostaglandins it is much more effective. Italian researchers (Barile et al., 2001) came to the same conclusion, emphasizing the major economic impact of such protocols in buffalo heifers and pointing out that at earlier age the effect is relatively better due the usual lack of cyclicity at that stage of individual development. All these effects are implied also in the results from the Bulgarian Murrah heifers and buffalo cows from the present experiment.

The integral role of the timed components of the OvSynch+PRID protocol is described by the experiment of McDougall and co-researchers (McDougall et al., 1992) in dairy cows with the observation that increased concentrations of progesterone during the anestrous are important for incidence of estrus and for the luteal phase afterwards, hypothetically contributing for maturation of a dominant follicle and hence for prostaglandins secretion and ovulation. The combination with prostaglandins at the beginning of the protocol was established to induce follicular atresia and a new follicular wave also in buffaloes (Baruselli et al., 2007).

When buffalo reproduction is concerned, season should be always taken in consideration. Having been included in the linear model with classes of the factor specific for the bubaline species within the conditions of Bulgaria, the effect of season is commensurate with the experience in the Italian Mediterranean breed, where especially in the recent decade the experiments with gonadotropins, progesterone and other exogenous hormones have demonstrated similar pregnancy rates after out-of-season breeding as in the high breeding season (Carvalho et al., 2013, 2016; Baruselli et al., 2013); and also with the experience in Murrah breed in India (Kumar P. et al., 2016). The lack of significant differences in the success of TAI by season is actually a good news for the conditions in Bulgaria. It implies that the use of these protocols, and especially OvSynch+PRID, affords manipulation of the breeding season for better reproductive efficiency, hence profitability, of buffalo farming. It also contributes for better distribution of bulk milk throughout the year, in case there is misbalance between annual dynamics of production and prices of raw milk, as it is in Italy.

CONCLUSIONS

All three protocols tested in the present study show capacity to mitigate the impact of season on reproduction, which in practice can be used for overcoming the species-problematic seasonal anestrus, especially OvSynch+PRID. It can be summarized that in the Bulgarian Murrah buffaloes the follicular wave in response to the hormonal protocols is similar to that in other breeds in different conditions. Namely, a favorable effect from the application of OvSynch+PRID in both age groups was established, and also of OvSynch in heifers, as well as of poor results from PreSynch/OvSynch. In our study, the superiority of the OvSynch+PRID protocol – as compared to the other protocols and also to other studies – finds expression in the higher incidence of full estrus especially in the heifers, which predetermines the high conception rate, despite the relatively high percentage of animals without CSE. In this way, its use in field conditions finds justification despite its labor-intensive application in practice. In this context, the presence of cervical mucus during AI can be confidently used as an indicator for high pregnancy rates under the protocols OvSynch and OvSynch+PRID.

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THE EFFECT OF CLIMATE CHANGE ON THE REPRODUCTION SEASON OF THE KARAKUL OF BOTOȘANI BREED

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Abstract

Although climate change is a certainty in the current era, very few people are aware of the medium and long-term effects it may cause. Therefore, the purpose of this research was to assess the impact of climate change on the breeding season of Karakul of Botoșani ewes. The research relied on the analysis and interpretation of data concerning the time interval when the first ewes exhibited sexual heat and when over 50% of the total number of ewes assigned for mating were mounted, spanning each season between 2000 and 2022. The biological material studied consisted of a representative population of adult ewes belonging to the Botoșani Karakul sheep breed, located at the Research Station for Sheep and Goats Breeding, in Popăuți-Botoșani. The obtained data highlight that between 2000 and 2011, the first ewes were mounted at the end of August (when the temperature was around 15°C). For the reproduction seasons between 2011 and 2022, it is observed that the period in which the first ewes exhibit sexual cycles shifted towards the middle of September (when the temperature at 8 AM remained around 15°C). Moreover, the period when over 50% of ewes exhibited sexual cycles and accepted mating also shifted towards the latter part of September and the first half of October. Under these conditions, it can be said that the third heat cycle, in which the proportion of mounted ewes exceeded 95%, shifted towards the first half of November.

Key words: *climate change, Karakul, reproduction, sheep.*

INTRODUCTION

The current period is witnessing climate change as one of the most significant threats to the environment, social framework, and economy. Global warming is already a recognized phenomenon and will likely lead to major imbalances in the environment. It will also affect the production and reproduction performance of animals.

According to the sixth assessment report on climate change by the experts of the Intergovernmental Panel on Climate Change (IPCC), human-produced greenhouse gas emissions are responsible not only for the rise in global temperatures but also for substantial and rapid changes in the atmosphere, oceans, cryosphere, and biosphere. The range of global temperature increase caused by humans, starting from 1850, ranges regionally between 0.8°C-1.3°C. What is more concerning is that according to the IPCC report (AR6), it is anticipated that in the coming decades, climate change will affect all regions of the world. As the global temperature rises, extreme changes continue to intensify. Some studies indicate that with every additional 0.5°C increase in temperature, there are noticeable rises in the intensity and frequency of extremes with extremely high temperatures, including heatwaves, heavy rainfall, variations in air current intensity, and intensified meteorological droughts in some regions of the world (IPCC, 2023). In the case of a 2°C temperature increase compared to pre-industrial levels, extreme temperatures would more frequently surpass critical thresholds for agriculture and health.

Despite animals being an important resource for humans, it is observed that people pay too little attention to climate change and the negative effects that can occur in this domain. In animal husbandry, climate change represents an area that is very poorly researched. This is why very little is known about the interactions between the increasing variability of climate and other factors that can affect animal husbandry systems and development under future pedoclimatic conditions (Thornton et al., 2009).

The effects of global warming will not be negative everywhere in the world and in all agricultural sectors. According to an analysis by Thornton et al. (2007), a slight increase in crop productivity is forecasted for agriculture in medium to high latitudes for a local average temperature increase of 1-3°C. However, in these areas, frost, heatwaves, or heavy rainfall can negate the advantages of increased temperatures. It is believed that a temperature increment of 1-2°C would severely affect crop production, especially cereals, in lower altitude regions. The most affected areas will be those in the northern hemisphere, especially North America, Northern Europe, Northern Asia, and to a lesser latitude, regions such as Mediterranean basin countries, Central and Western Asia (Easterling et al., 2007).

To guide the evolution of animal production systems under the influence of temperature increase and extreme climatological events, better information regarding biophysical and social vulnerability is needed. This information should be integrated with future agricultural and animal husbandry components.

MATERIALS AND METHODS

The area where the research was conducted is situated in the northeastern part of Romania, at 47°44′55 north latitude and 26°40′10 east longitude. This specific area is significantly affected by climate change, particularly the extension of periods with higher temperatures and extremely reduced precipitation.

The research began by observing that in recent times, with atmospheric temperatures frequently exceeding the thermal comfort limit, there has been a considerable change in the onset and execution of mating in Botoșani Karakul sheep breed ewes.

Presumably, these changes in climate are at the root of this situation, generating negative effects on the animals' bodies, affecting their reproductive activity and ovine performances.

To gather accurate information about the impact caused by climate changes on the breeding season, data were collected over a longer period, specifically spanning 20 years. Based on this data, the onset of the breeding season was determined, as well as the calendar interval in each season when the highest proportion of mounted ewes occurred.

All the data used in this study originate from farm records located at the Research and Development Station for Sheep and Goats Breeding, in Popăuți-Botoșani. Specific temperature data for each mating season were obtained from local meteorological stations.

RESULTS AND DISCUSSIONS

Climate change is an undeniable aspect, yet its effects are often controversial. Therefore, the aim of this research was to evaluate the effects caused by climate change on the reproductive activity within a population of sheep belonging to a breed that holds significant importance in the breed structure within the Northeast Region of Romania.

In the unit where the research was conducted, the breeding season traditionally started in the third decade of August and ended in the first half of October. However, in recent seasons, it has been observed that the breeding season's onset has shifted to late September and concluded in the second half of October.

To analyze the data and the conditions under which the reproductive activity occurred, all information regarding the day when the breeding season commenced was extracted from farm records over a representative time interval from 2000 to 2022. Based on these records, the timeframe in which at least 50% of the total allocated females were mated each season was also determined (Table 1 and Table 2).

Additionally, for this timeframe, data on the average values of daytime temperatures recorded at 8 AM on each day of the initial pairings were extracted. The obtained data highlight both consistency in the thermal regime and certain seasonal differences, for the day when the first ewes exhibited the sexual cycle and for the interval in which over 50% of the total ewes assigned for mating were mounted each year (Figure 1).

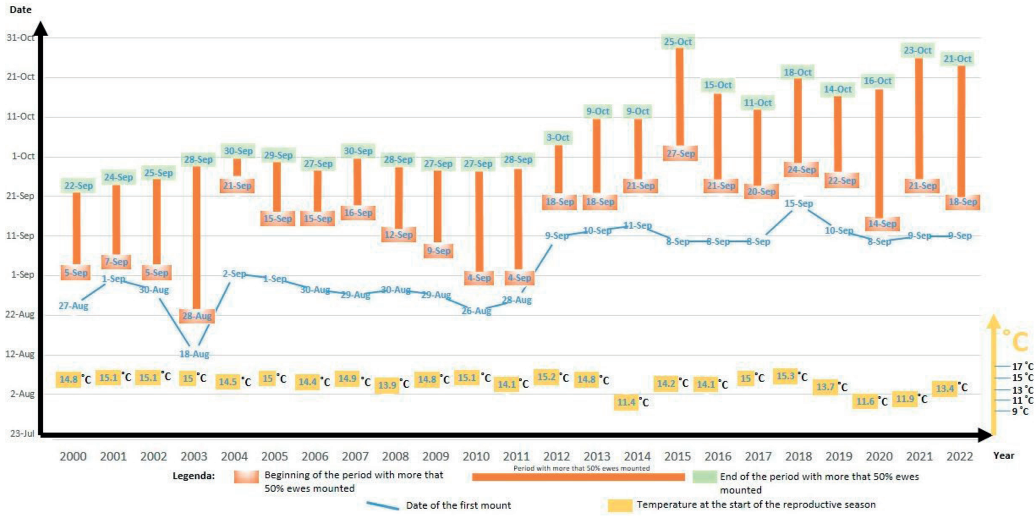

Figure 1. Graphical distribution of temperatures and the onset and end range of ewes' mating season

For the interval between 2000 and 2011, it was observed that the first pairings occurred at the end of August; an exception was noted in the seasons of 2004 and 2005 when the first females exhibited sexual cycles at the beginning of September (under similar thermal conditions to the analyzed time frame).

The analysis of the thermal regime corresponding to the periods of each year when the first females were inseminated highlights that the recorded values at 8 AM fluctuate within a relatively narrow thermal range, between 13.9°C and 15.1°C (Figure 2).

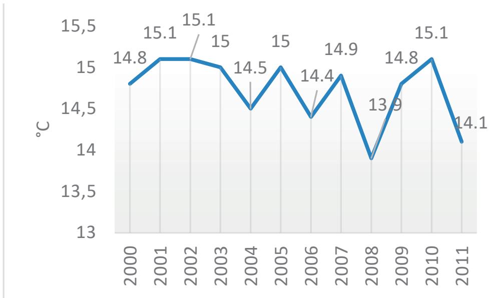

Figure 2. The distribution of the thermal regime when the reproductive activity started in Botoșani Karakul sheep breed

Table 1. The debut of breeding season and the time interval in which >50% of the ewes allocated for mating were inseminated between 2000-2011

Year	The debut of breeding season		The calendar interval during which >50% of the ewes were mated
	The date of first breeding	The temperature at 8 AM (˚C)	
2000	27.08	14.8	05.09 - 22.09
2001	01.09	15.1	07.09 - 24.09
2002	30.08	15.1	05.09 - 25.09
2003	28.08	15.0	18.08 - 28.09
2004	02.09	14.5	21.09 - 30.09
2005	01.09	15.0	15.09 - 29.09
2006	30.08	14.4	15.09 - 27.09
2007	29.08	14.9	16.09 - 30.09
2008	30.08	13.9	12.09 - 28.09
2009	29.08	14.8	09.09 - 27.09
2010	26.08	15.1	04.09 - 27.09
2011	28.08	14.1	04.09 - 28.09

For the time interval between 2000 and 2011, it is also observed that the time boundaries within which mating occurred with a proportion greater than 50% of the number of females allocated for mating, fall in the second half of September each year. In practice, each year, this interval corresponds to the second heat cycle, meaning that the last pairings occurred during the third cycle and concluded in the first half of October.

For the analysis period between 2012-2022, a shift is noticed in the time interval when the first pairings occurred (Table 2). Examining the data related to this aspect reveals that after 2011, there is a shift towards the middle of September regarding the first day when the first adult ewes showing clear signs of entering heat were identified.

Table 2. The debut of breeding season and the time interval in which >50% of the ewes allocated for mating were inseminated between 2012-2022

Year	The debut of breeding season		The calendar interval during which >50% of the ewes were mated
	The date of first breeding	The temperature at 8 AM (˚C)	
2012	09.09	15.2	18.09 - 03.10
2013	10.09	14.8	18.09 - 09.10
2014	11.09	14.1	21.09 - 09.10
2015	08.09	14.2	27.09 - 25.10
2016	08.09	14.1	21.09 - 15.10
2017	08.09	15.0	20.09 -11.10
2018	15.09	15.3	24.09 - 18.10
2019	10.09	13.7	22.09 - 14.10
2020	08.09	13.6	14.09 - 16.10
2021	09.09	13.9	21.09 - 23.10
2022	09.09	13.4	18.09 - 21.10

Throughout the analyzed interval, it is observed that the first pairings occurred between September 8th and 15th. After 2011, there is a tendency noticed for a shift of approximately 14 days in the date when the mating season begins (Table 2).
The data regarding the temperature levels recorded at 8 AM for each day when the first ewes were mated indicate that the average values fall within the same range, ranging between 13.4˚C and 15.3˚C (Figure 3). This aspect was possible because the period when optimal temperatures for triggering heat in sheep shifted towards the second half of September.

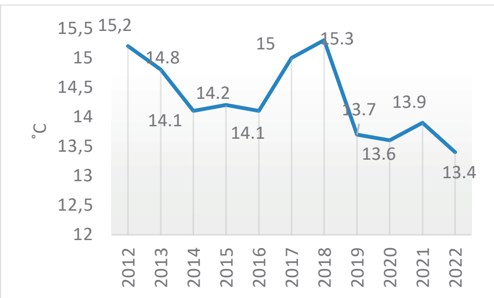

Figure 3. The distribution of the thermal regime when the reproductive activity started in the Botoșani Karakul sheep breed

Furthermore, it is observed that the period during which over 50% of ewes exhibit heat and accept mating has shifted towards the second part of September and the first half of October. Under these circumstances, it can be said that the third heat cycle, during which the proportion of sheep mated in that season exceeds 95%, has shifted towards the first half of November.
Climate change refers to a modification in the climate state that can be identified and persists for an extended period, usually for several decades or more (UNFCCC 2011). Some studies indicate that between 1901 and 2007, the average annual air temperature in Romania increased by 0.5°C (Climate Regions, 2011).
Similarly, data obtained from 94 meteorological stations show significant changes in seasonal average temperatures between 1961-2007. Specifically, during this interval, there was an approximately 2°C increase in average temperatures during summer, winter, and spring, with a slight tendency for a decrease in average temperatures during autumn (MEF, 2010). Other sources present similar findings concerning various temperature extremes, such as daily maximum and minimum temperatures (Busuioc et al., 2015), the number of summer days with temperatures outside comfort limits (Dobrinescu et al., 2015).
Climate change is expected to intensify in the coming years, continuing the trend observed in recent decades. According to the National Meteorological Administration of Romania, in 2021, the average annual temperature was recorded at 10.90°C, with a thermal deviation of 0.69°C compared to the 1981-2020 average, confirming the evident trend of rising air temperatures in recent decades.
According to NMA Romania, positive thermal anomalies for the 2012-2021 period ranged between 0.69°C (2021) and 1.92°C (2019), marking the hottest period of ten consecutive years in the history of meteorological measurements, attributed to climate warming. In descending order, the warmest years in the 2012-2021 period were: 2019, 2020, 2015, 2007, 2018, 2014, 1994, 2009, 2013, 2012, and 2021 (NMA Romania).
Consistent with these values, there is a trend of significant temperature increase for all seasons in recent times, except for autumn, with the highest rate of increase in summer and the lowest in spring (Busuioc et al., 2015).
The increase in average temperatures beyond certain thresholds has been relatively constant in the last 40 years across Europe.

Climatological data analysis for Romania shows a trend of increasing multiannual average temperatures over the past century (Figure 4). Additionally, the research area not only shows a tendency of rising average temperatures but also a reduction in atmospheric precipitation (Figure 5).

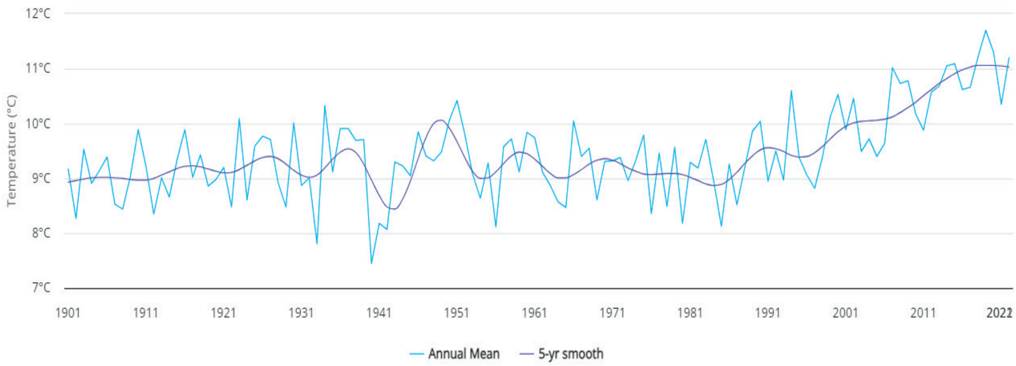

Figure 4. The evolution of multiannual average temperatures in Romania (1901-2022)

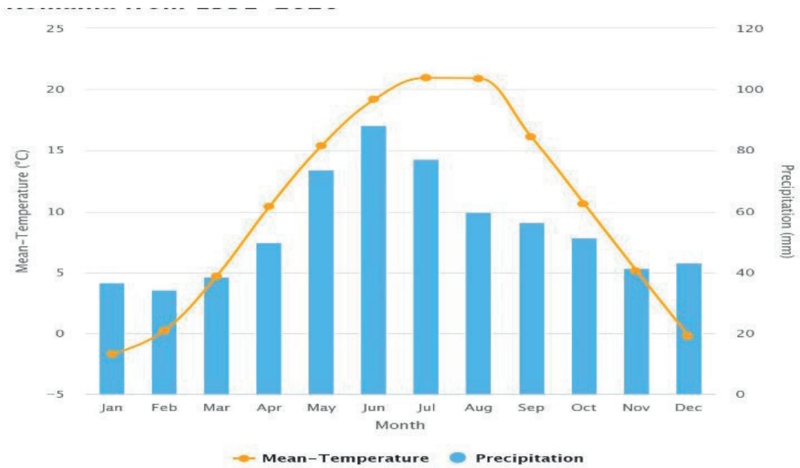

Figure 5. The average monthly temperatures and precipitation patterns in Romania (1991-2020)

Climatological diagrams based on data collected from six weather stations in the North-Eastern Region of Romania indicate a significant reduction in the period when both daytime and nighttime temperatures drop below 0°C, and dry periods have extended in the last 30 years due to climate change.

Air temperature in Botoşani has experienced a remarkable increase statistically significant from 1961 to 2017. The average annual temperature increased by 0.31°C per decade or 1.8°C throughout the period, with the maximum statistical significance ($p < 0.001$). Warm and cold seasons, summers, springs, winters, and the months of January to August notably show a statistically significant increase in air temperature (Strătilă et al., 2021).

Since animal reproduction, including sheep, is a complex process involving various physiological and psychological aspects governed by hormones, metabolites, and environmental factors, it's clear that climate change can lead to certain modifications. Ewes and rams are highly sensitive to high temperatures and undernutrition (Nechifor et al., 2022; Simeanu et al., 2023; Staykova et al., 2023; Pascal et al., 2023).

Continuous exposure of animals to thermal stress compromises growth, milk and meat production, and reproduction. An animal's capacity to mitigate the effects of rising environmental temperatures without progressing towards acquiring stress resistance differs within and between species (Joy et al., 2020).

All reproductive processes, such as gametogenesis, puberty, gamete migration, fertilization, early embryonic development, maternal recognition of pregnancy, gestation, birth, and postpartum recovery, can be indirectly influenced by environmentally-induced stress and directly affected by impairing the functions of reproductive organs or by blocking hormone-mediated cellular functions of the hypothalamic-pituitary-gonadal axis (Florea et al., 2017; Florea et al., 2021; Kumar et al., 2017; Pascal et al., 2008).

Increasing air temperatures cause animals to experience longer periods of heat stress, affecting both rams and ewes. For rams, the increment of body temperature during breeding period stress leads to testicular degeneration, a decrease in the percentage of normal and fertile sperm, reduced ejaculate volume, higher sperm pH, decreased sperm motility, and reduced sperm quality (Hamilton et al., 2016; Rahman et al., 2016). For ewes, exceeding specific thermal comfort limits increases the risk of mammary gland infections in lactating ewes (Koyuncu et al., 2018), as well as reducing lamb birth weights and viability during both colder and hotter months (Luo et al., 2020).

To mitigate the impact of stress factors, farmers need to adapt to current circumstances and adopt technologies and management practices that limit the negative effects of climate change; otherwise, substantial losses may occur.

Losses will arise because the animal environment is affected by key climatic factors (temperature, humidity, radiation, and wind). In any of these situations, extreme climatic values induce a change in energy transfer between the animal and its environment, potentially affecting reproductive activity. Seasonal variations in the environment, alongside nutrition and management, can also lead to changes in estrous activity and its duration. Moreover, it's known that conception rates are reduced under heat and cold stress, and endocrine functions are altered by climatic extremes (Gwazdauskas, 1985).

Regarding sheep, Narayan et al. (2018) specify that heat stress reduces embryo production during artificial insemination or embryo transfer due to disruptions in essential physiological elements, affecting not only reproductive function but also early embryo development in pregnant ewes.

Climate change and the increase in days when temperatures exceed normal limits can diminish the reproductive capacity of ewes because physiological adaptations struggle to cope with heat stress (Hansen, 2007a; Hansen, 2007b).

Extreme climatic conditions are undesirable as they can trigger physiological, biochemical, hematological, and hormonal changes that can affect the active maintenance of homeothermy and the productivity of sheep. Knowing the comfort limits and ensuring constant optimal values are challenging to manage during the warm season. Under these conditions, heat stress occurs, leading to disturbances in mechanisms responsible for heat stress tolerance in animals (McManus et al., 2020).

Apart from the effects of climate change on ecosystems, it's evident that prolonged periods of high temperatures will significantly alter the natural resources essential for animal production. Climate features such as temperature and precipitation levels have a significant impact on pasture productivity and other resources, affecting the ruminants' growth process (Tüfekci et al., 2021).

Several studies demonstrate that climate change and its disruptive factors can affect sheep in two ways: directly by reducing lamb survival rates and indirectly by influencing the vegetation season, endangering net primary productivity of pastures and forage availability (Castillo et al., 2021).

CONCLUSIONS

Climate change, mainly due to rising temperatures, affects the reproductive activity of Botoșani Karakul sheep breed.

Since 2011, the period when over 50% of ewes exhibit heat and accept mating has shifted towards the latter part of September and the first half of October.

This alteration of the period of sexual cycles manifestation also leads to a shift towards early November when over 95% of ewes forming the livestock exhibit sexual cycles and become pregnant.

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STUDY ON THE PHYSICOCHEMICAL PROFILE OF COLOSTRUM FROM ANGLO-NUBIAN GOATS 24 HOURS AFTER PARTURITION

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Abstract

The specific properties of colostrum make it an indispensable source of nutrients and passive immunity for newborns. Feeding kids with colostrum within the first hours after parturition is extremely important for their health and survival. The composition of colostrum in different animal species is not the same. There is also a difference in terms of interbreeding. The present study aims to monitor the changes in the physicochemical parameters of colostrum from Anglo-Nubian (AN) goats that occurred within the first 24 hours after parturition. The study was conducted in the goat farm of the RIMSA, Troyan, Bulgaria, and a total of 40 colostrum samples were obtained from clinically healthy AN goats. For the studied period of 24 hours, a reliable decrease of the studied parameters was reported: protein (14.21-7.79%), total solids (24.02-20.84%), solid-not-fat (19.21-12.93%), density (1.045-10.354 g/ml^3), acidity (32-21^{o}T) and Ca (0.254-0.1494 mg/%). In contrast, for the indicators, such as fat (5.32-8.37%), lactose (2.63-3.43%), and pH (6.24-6.31), an increase in the values was observed.

***Key words**: Anglo-Nubian breed, colostrum, goats, physicochemical indicators.*

INTRODUCTION

Feeding colostrum within the first hours after parturition is especially significant for the health and survival of the kids. It is the first secretion that is released from the mother's udder after parturition (Agradi et al., 2023), different in appearance and content from usual milk, and it has a thicker consistency than milk, and is yellowish (Dimov et al., 1975, Kıvrak et al., 2012, Anitaş & Göncü, 2024,).

Changes in the composition and properties of colostrum are most significant during the first day after parturition (Sánchez-Macías et al., 2014). During the so-called "transition period", or in other words, the transition of colostrum into milk, changes are observed in its biochemical and physiological indicators (Arain et al., 2008; Övet, 2023).

Both interspecies and interbreed differences in the colostrum composition have been found. This question is reported by Koluman et al. (2019) (comparing cow, sheep, and goat colostrum), Anitaş et al. (2021) (in Awassi and Çukurova Meat Type Sheep Breeds), Kumar et al. (2014) (in Sannen x Beetal and Alpine x Beetal goats) and others.

König (1903) and Bergman & Turner (1936) cite Henry (1840), who first made studies on the goat colostrum composition. According to Arguello et al. (2006), the colostrum composition varies among goat breeds. Information on the quality of colostrum from Anglo-Nubian goats, and its physicochemical and immunological properties is limited.

This information would allow a more accurate assessment of proper suckling, health status, and to what extent the newborn has received passive immunity from the mother. That is why that paper is focused on the physicochemical profile of colostrum from Anglo-Nubian goats raised in Bulgaria, as well as its change within the first 24 hours after parturition.

The present study aims to observe the changes in the physicochemical parameters of colostrum from Anglo-Nubian (AN) goats that occurred within the first 24 hours after parturition.

MATERIALS AND METHODS

The study was conducted in the goat farm of the Experimental Base of the Research Institute of Mountain Stockbreeding and Agriculture of

Troyan, located on the foot-hill of the Central Balkan Mountain, at 380 m above sea level.
The colostrum analyzed for the present study was obtained from twenty clinically healthy Anglo-Nubian goats during the period of kidding. The samples were milked at the 1st and then at the 24th hour after parturition, as each of them was placed in an individual container of 200 ml and transported to the Meat and Milk Laboratory at the laboratory complex of RIMSA-Troyan.
The physicochemical analysis includes a total of nine indicators, such as milk fat, protein, lactose, solids, and solid-not-fat, which were analyzed on a MilkoScan FT 120 Foss Electric device, and the samples were previously tempered to 40°C in a water bath to homogenize them. The obtained results are expressed in percentages (%).
Acidity and Calcium (Ca) were determined by standard titration methods.
Colostrum density was measured by weight method, at a sample temperature of 20°C, and reported in g/cm^3.
Active acidity or pH was determined using a pH meter 3110 SET 2- Wissenschaftlich Technische Werkstatten.
JMP v7 software package was used for the statistical processing of the results.

RESULTS AND DISCUSSIONS

Table 1 shows the chemical composition of colostrum at both time points it was obtained.

Table 1. Chemical composition of colostrum from AN goats within 24 h postpartum, %

Indicators (%)	1 h				24 h				Significance
	Mean	Under	Upper	SD	Mean	Under	Upper	SD	1 h x 24 h
Fat	5.32	4.53	6.11	0.27	8.37	7.58	9.16	0.48	***
Protein	14.21	12.8	15.61	0.88	7.79	6.39	9.2	0.43	***
Lactose	2.63	2.28	2.98	0.22	3.43	3.08	3.78	0.1	**
TS	24.02	22.27	25.78	1.1	20.84	19.08	22.59	0.54	*
SNF	19.21	17.69	20.74	0.98	12.93	11.41	14.46	0.41	***

**** p < 0.001; ** p < 0.01; *p < 05*

Protein

Within the 24-hour postpartum period (Table 1), the amount of protein in the colostrum studied by us decreased by 6.42% ($p < 0.001$). The percentage decrease of this indicator is higher compared to what we found in the colostrum of Toggenburg goats (Mondeshka & Stoycheva, 2023), which is 3.81% ($p < 0.001$) and lower than what was found in the goat colostrum from Bulgarian White Dairy breed with 9.64% for the same period (Stoycheva & Mondeshka, 2023).
In Murciano-Granadina goats, Romero et al. (2013) found similar results to ours for protein, 13.64% at the 1st hour and 6.24% at the 24th hour postpartum, the difference between the two values (7.4%) is close to ours and confirms the trend to a significant decrease in the protein amount in the colostrum during the first day after parturition.
Anitaş et al. (2021) reported 18.60 ± 0.31% protein in the Awassi sheep colostrum and 10.90 ± 3.16% in the colostrum of Çukurova Meat Type Sheep Breed obtained at 16 h postpartum. Kessler et al. (2019) reported 16.36% protein in the colostrum of Anglo-Nubian goats raised in Switzerland and Germany obtained within the first two hours after parturition.
The decrease in protein content in colostrum found in many breeds could be due to the large decrease in the number of immunoglobulins, leukocytes, lactoferrin, lysozyme, growth hormones, some amino acids, etc. within the 24 hours after parturition (Pellegrini et al., 1994; Rashid et al., 2012).

Lactose

Lactose is the main carbohydrate that newborns receive with colostrum. Also called milk sugar, it is a disaccharide found only in milk, milk products, and nowhere else in nature. Lactose has been found to stimulate the synthesis of B vitamins (Dimov et al., 1975). Lactose favors the intestinal absorption of calcium, magnesium, phosphorus, and vitamin D3 (Chilliard et al., 2003; Rashid et al., 2012). Kračmar et al. (2002) summarized the research of Hadjipanayiotou (1995) who compared milk and colostrum from sheep, goats, and cows and reported that the amount of lactose increased sharply in samples taken consecutively during the colostral period and remained relatively high throughout the transition and lactation period.

In the present study, an increase was observed in the milk sugar content in the colostrum from 2.63 to 3.43% ($p < 0.001$) within the studied 24-hour period (Table 1). The present finding is in agreement with many researchers, such as Kračmar et al. (2002) in Brown Short-haired breed goats; Sánchez-Macías et al. (2014) in Majorera goats; Rashid et al. (2012) in Beetal goats etc.

Fats

The percentage fat content of colostrum from Anglo-Nubian goats found in the present study was 5.32% at the 1st hour after parturition and 8.37% at the 24th hour (Table 1). Some publications present differences, which are determined by the breed, in the dynamics of this indicator within the first 24 hours after parturition. The fat and lactose in the colostrum are required by the newborn for heat production and the prevention of hypothermia (Keskin et al., 2007).

In domestic breeds of Romanian goats, Zaharia et al. (2011), investigated changes in fatty acid composition and cholesterol content of colostrum and followed the change in fat content from 0 to 7 days. The results published by the author's team, regarding the fat content, were obtained from samples taken for examination on 0 h - 4.20%; 6 h - 8.08%; 12 h - 5.14%; 24 h - 6.02%, and 48 h - 4.80%.

It is obvious that the results are quite dynamic and represent alternating increases and decreases in the percentage of lipids in the colostrum samples. Arguello et al. (2006) analyzed the effects of lactation sequence and number of kids born on the physicochemical parameters of colostrum from Majorera goats and found an increase in fat during the first day after parturition and a subsequent decrease until 132 hours after parturition. Marounek et al. (2012) examined colostrum from three-year-old White Shorthaired goats and found a decrease in fat from 5.67% at the 1st hour to 4.11% at the 24th hour.

Rashid et al. (2012) reported a much lower fat content in Beetal goats at the 1st day with 3.8%, followed by a continuous increase at the 2nd (4.5%) and 3rd day (5.2%). According to Dimov et al. (1975), the amount of fat in milk varies depending on several factors, such as the lactation period, the season, and above all, nutrition - the type and ratio of forage used for animal feed.

The solid-not-fat (SNF)

In the present study, SNF of colostrum decreased by 6.28% ($p < 0.001$) at the 24th hour compared to the 1st hour after parturition (Table 1). The decrease is a logical consequence of the changes that occur when the other investigated colostrum indicators are changed. Proteins, lactose, and minerals make up the solid-not-fat. It is a relatively more constant quantity than total solids (Dimov et al., 1975).

The decrease mentioned above is a logical consequence of the dynamic changes occurring within the first hours after parturition, in the other physicochemical parameters of colostrum, since there is a certain dependence between the percentage content of SNF and proteins, lactose, and mineral substances. SNF is a relatively more constant quantity than total solids (Dimov et al., 1975).

Our findings correspond to the results of other authors on different goat breeds, such as Sánchez-Macías et al. (2014) for Majorera goats, Marounek et al. (2012) for White shorthaired goats, etc.

According to Prasad et al. (2002) the higher the solids content of the colostrum, the higher the density. Kracmar et al. (2005) and Hadjipanayiotou (1995) also described a higher content of total solids in colostrum immediately after parturition and a subsequent decrease. A decrease in colostrum density values within the first 24 hours after parturition was registered (Figure 1).

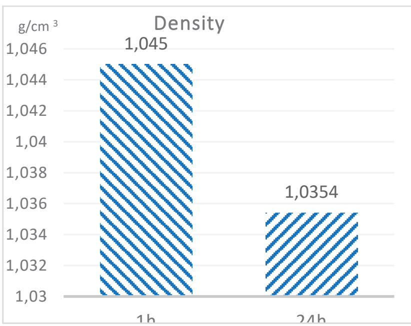

Figure 1. Mean density values (g/cm^3) of colostrum from AN goats at 1st h and 24th h after parturition

The **total solids** in the present study decreased from 24.02% at the 1st hour to 20.84% at the 24th hour after parturition (Table 1).
Keskin et al. (2007) reported that the total solids content of colostrum of Damascus goats was the highest on the 1st day and decreased gradually until the 3rd day. The present data correspond to those presented by Romero et al., (2013) for Murciano-Granadina goats and Tôrres Vilar et al. (2008) for Saanen goats.

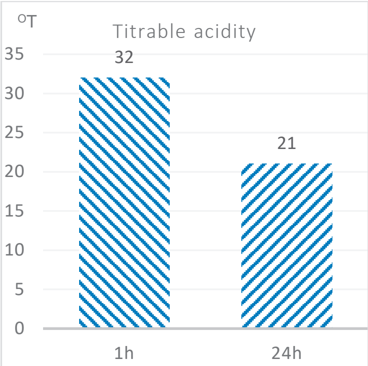

Figure 2. Mean values for the titratable acidity (ºT) of colostrum from AN goats at 1st h and 24th h after parturition

Titratable acidity is determined immediately after milking and indicates the presence of phosphates, citrates, proteins, and gases in milk. According to Dimov et al. (1975), it is influenced by nutrition, the health status of the animal, and the lactation period, as these factors probably also influence the colostrum composition (Tôrres Vilar et al., 2008).
We found values of 32ºT at the 1st hour after parturition and 21ºT at the 24th hour (Figure 2). In previous studies, we found 35ºT at the 1st hour after parturition and 20ºT in the 24th h in Bulgarian White Dairy goats (Stoycheva & Mondeshka, 2023) and values of 25ºT at the 1st hour after parturition and 21ºT in the 24th h in Toggenburg goats (Mondeshka & Stoycheva, 2023). Torres Vilar et al. (2008) reported acidity of 46.1 ºD at the 1st hour and 25.1 ºD at the 24th hour in Saanen goats, which was higher than what we found, but the trend was similar.

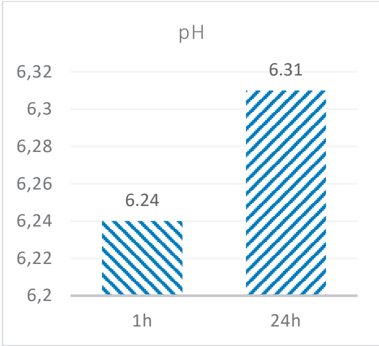

Figure 3. Mean pH values of colostrum from AN goats at 1st h and 24th h after parturition

The values of active acidity **(pH)** of the colostrum we studied increased from 6.24 at the 1st hour to 6.31, 24 hours later (Figure 3).
In Murciano-Granadina goats, Romero et al. (2013) reported values for this indicator from 6.38 at the 1st hour to 6.58 in the 24th h. Similar results to ours were also reported by Tôrres Vilar et al. (2008) in Saanen goats and Sánchez-Macías et al. (2014) in Majorera goats Some authors consider that the change of active acidity is influenced by the number of lactations of the goat (Tôrres Vilar et al., 2008, Romero et al., 2012), whereas others do not find such a dependence (Argüello et al., 2006).
At the 1st hour after parturition, we found Ca content of 0.254 (mg/%), in the colostrum obtained from Anglo-Nubian goats, as the value of this indicator was 0.194 (mg/%) at the 24th hour (Figure 4).

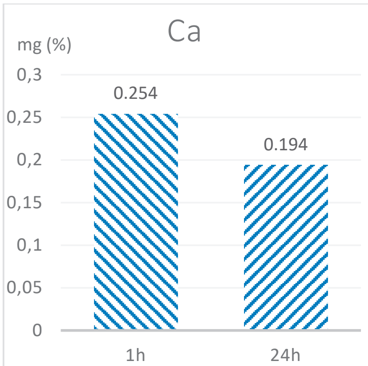

Figure 4. Average Ca values (mg/%) of colostrum from AN goats at 1st h and 24th hour after parturition

The calcium content in fresh goat's milk is found in the ionic, molecular, and colloidal-dispersed state. Its amount in milk is relatively constant. The increased or decreased content of calcium in milk depends on the health of the animals, the lactation period, and their nutrition (Dimov et al., 1975; Pecka-Kiełb et al., 2018). Hamed et al. (2023) reported a decrease in the amount of Ca when the colostrum transits into milk for Baladi goats, from 511.695 ± 0.22 to 27.924 ± 0.21 mg/100 g.

CONCLUSIONS

In conclusion, the present study showed the change in the colostrum composition obtained from Anglo-Nubian goats within the first 24 hours after parturition, which is within the optimal limits for this type of animal.

A reliable increase in the values of fat, lactose, and pH, and a decrease in the values of protein, total solids, solid-not-fat, density, acidity, and Ca were found.

The results of the present study could be used as a basis for interbreed differentiation and criteria for determining the main quality characteristics of colostrum from Anglo-Nubian goats at the 1st day after parturition, which is decisive for the survival of the newborns and their normal development.

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STUDY REGARDING THE INFLUENCE OF THE AGE OF FIRST CALVING, PARITY, NEW-BORN WEIGHT AND CALVING SEASON ON DAILY GAIN OF CALVES GROWTH IN A FARM IN NORTH-EAST OF ROMANIA

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Abstract

The aim of the present study was to highlight the influence of the age of first calving, parity, new-born weight and calving season on the average daily gain. The biological material consists of Holstein calves reared in an intensive system. The data obtained from the measurements at 7 days and 30 days, were statistically processed with the help of Statistics Analysis of Variance and Covariance (SAVC) and SPSS 16.00 computer programs. Significant differences between batches ($p<0.05$) were observed at 7 days, between calves born by cows having second parity which has a mean with 0.300 g higher of daily gain than those calved by third parity cows. At 30 days, the daily gain mean value has significantly greater differences ($p<0.05$), been with 0.12 g/day higher in the case of calves born in winter compared to those born in summer and with 0.14 g/day compared to those calved in spring ($p<0.01$). In the case of calves studied for age at first calving and birth weight, there are no differences between the batches so, these factors not influencing their further development in the case of the considered periods.

Key words*: age at first calving (AFC), daily gain, Holstein, parity.*

INTRODUCTION

For the calves, the suckling period is a critical one in terms of the future performance and the evaluation of the body development of the animals is done by monitoring the daily weight gain (DWG). Studies show an association between body weight achieved at 30 days and a reduced age at first calving (Brickell, 2009; Raducanu et al., 2023) and that DWG influence the productive performance in first lactation. Thus, daily weight gain of more than 500 g/day during this period has a positive impact on production in first lactation and some studies has suggest that each 100 g of DWG can be associated with an extra 85-110 kg of milk. (Hyde M.R. et al., 2021).

The evolution of calf weight gain is influenced by a number of different factors such as genetic (breed) or non-genetic ones (age at first calving of the female, parity, calving and breeding season, etc.) (Shivley et al., 2018). The value of the daily weight gain is influenced by factors related to management practices such as the administration of colostrum, the amount of milk administered (Wieland et al., 2017; Tautenhahn et al., 2020) and its composition, climatic factors and microclimate, therefore it is necessary a holistic approach to them. The most important influencing factor during this period is nutrition, calves fed *ad libitum* with milk gained four times more than restricted-fed calves, which also showed signs of starvation (De Paula Vieira et al., 2008; Nica et al., 2023).

The hygiene of the bedding and the individual bunks as well as the group housing of the calves are organizational measures that indirectly improve the rate of growth by reducing the incidence of diseases (Horvath & Miller-Cushon, 2018; Lindner et al., 2021).

MATERIALS AND METHODS

The present study aims to highlight the influence of some factors such as age at first calving (AFC), female parity, birth weight (BW) of calves and calving season on the daily weight gain (DWG) of calves.

DWG was calculated at 7 and at 30 days age, for a batch of 154 calves of the Romanian Black and wihte breed (Holstein strain), males and females, calved between June 2022 and July 2023. The analyzed batches were obtained after excluding individuals that they had digestive, respiratory or other pathologies during the reference period, causes that could have affected the growth rate of the animals. The results of the measurements were grouped according to the influencing factors followed, namely the age at first calving (AFC) and the parity of the cow, the birth weight (BW) of the calves and the calving season.

In the studied farm, calves are rearing in individual banks and during the first five days of life are fed with quality colostrum (≥ 30 °Brix) from the cows - mother or foster-mother. After the end of the colostrum period, they are fed daily with a quantity of 8 liters of whole milk distributed in two meals. A first assessment of body development was made 7 days, after the end of the colostrum period and then at 30 days. Weight assessment was done indirectly, based on chest circumference measurements and the observed values were entered into the eWeigh application for cattle. Based on the assessed weight, the average daily gain at 7 days (DWG7) and at 30 days (DWG30) were calculated.

The primary data were statistically processed with the help of the SAVC (Statistics Analysis of Variance and Covariance) software, respectively SPSS 16.00 for WINDOWS. Thus, a series of statistical estimators were determined such as: the arithmetic mean ($\overline{X}$), the standard deviation of the arithmetic mean ($\pm s_{\bar{x}}$), the standard deviation (s), the coefficient of variability (V%), the Fisher Test and the Tukey Test, the significance test p. and the confidence interval (C.I.) (Cucu et al., 2004).

RESULTS AND DISCUSSIONS

The mean value of DWG7 in studied batch is 0.744 kg and for DWG30 it is 0.578 kg. For age at first calving, data on DWG7 and DWG30 values were grouped into three classes, namely: cows whose age at first calving was up to 800 days (approx. 26 months), those that had AFC between 801-860 days (26-28 months) and over 860 days.

Table 1. Statistical estimators for DWG (kg) depending on cows` age of first calving

Treats		n	$\overline{X}$	$\pm s_{\bar{x}}$	s	V%	Significance
AFC < 800 days	DWG7	74	0.750	0.052	0.443	59.339	n.s.
	DWG30	82	0.580	0.02	0.18	31.012	n.s.
AFC 801-860 days	DWG7	27	0.830	0.083	0.433	51.897	n.s.
	DWG30	36	0.590	0.028	0.169	28.817	n.s.
AFC > 860 zile	DWG7	20	0.730	0.058	0.258	35.256	n.s.
	DWG30	24	0.560	0.038	0.187	33.344	n.s.

n.s. - non-significant

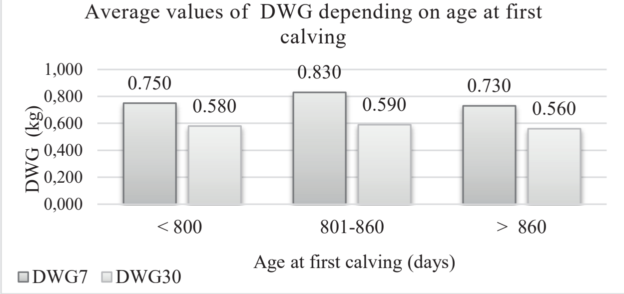

Figure 1. Mean of DWG depending on cows` age of first calving

From the analysis of the data summarized in Table 1 and the graphical representation in Figure 1, it can be seen that the highest mean values for both DWG7 and DWG30 were recorded in the calves of cows having AFC of 26-28 months. For the calves of these females the average AWG7 is 0.870 kg and the average AWG30 is 0.590 kg. Character variability is high, the calf categories considered being very heterogeneous in terms of weight gain. By applying the Tukey test, no statistically significant differences were found between the samples ($p < 0.05$, CI = 0.95)

Table 2. Statistical estimators for DWG (kg) depending on parity

Traits		n	$\overline{X}$	$\pm s_{\bar{x}}$	s	V%	Significance
First calving	DWG77	61	0.74	0.054	0.424	57.304	n.s.
	DWG30	69	0.574	0.025	0.205	35.620	n.s.
Second calvin	DWG77	33	0.93	0.08	0.459	49.269	*s.
	DWG30	38	0.593	0.025	0.156	2.306	n.s.
Third calving	DWG77	25	0.63	0.071	0.357	56.372	*s.
	DWG30	31	0.555	0.024	0.132	23.836	n.s.
Fourth calving	DWG77	6	0.78	0.089	0.219	28.170	n.s.
	DWG30	8	0.617	0.056	0.157	25.490	n.s.

*s - significant ($p < 0.05$); n.s. - non-significant

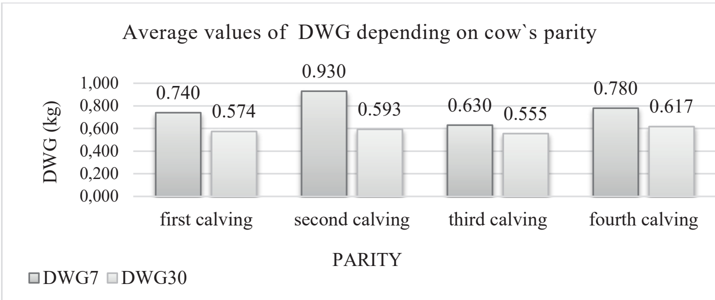

Figure 2. Average values of DWG depending on cow`s parity

In Table 2 are displayed the calculated values of the statistical estimators of DWG7 and DWG30 according to cow parity. As can be seen also in Figure 2, the highest average value of 0.930 kg of DWG7 was recorded in the calves resulting from the second calving and in the case of DWG30 the highest value of the daily growth increment is 0.617 kg in the calves from a fourth calving. The DWG7 average of 0.780 kg is also good in the case of calves from the fourth calving, the variability of trait in this case being medium homogeneous compared to the other categories characterized by heterogeneity. Statistically significant differences ($p < 0.05$, CI = 0.95) were found between the DWG7 of calves calved by cows on second calving having a mean value of 0.300 kg higher compared to that of calves from third calving dams.

Table 3. Statistical estimators for DWG (kg) depending on calves birth weight

Caractere		n	$\overline{X}$	$\pm s_{\bar{x}}$	s	V%	Significance
BW 30-40 Kg	DWG7	19	0.91	0.121	0.528	57.877	n.s
	DWG30	21	0.61	0.034	0.157	25.917	n.s
BW 40-50 Kg	DWG7	92	0.75	0.036	0.35	46.601	n.s
	DWG30	101	0.57	0.015	0.146	25.475	n.s
BW > 50 Kg	DWG7	21	0.74	0.121	0.555	75.415	n.s
	DWG30	32	0.58	0.047	0.264	45.864	n.s

n.s. - non-significant

The birth weight of calves is another factor considered for possible influence on DWG. Table 3 shows the calculated values of the estimators, in this case the heterogeneity of the character can be observed for all weight categories of the calves, the V% coefficient having the lowest value of 25.5%.

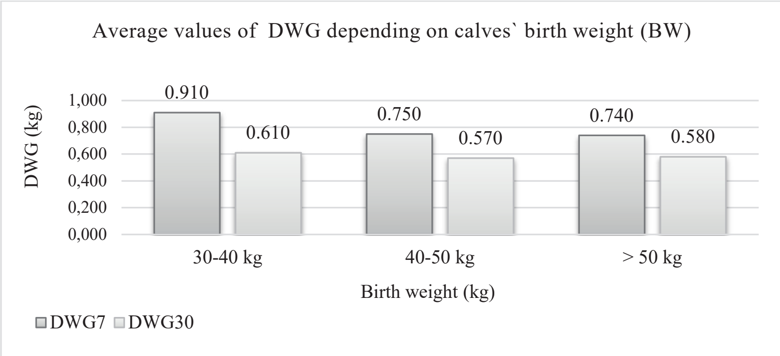

Figure 3. Average values of DWG depending on calves` birth weight

The graphic representation in Figure 3 of the values of the arithmetic means highlights the fact that the greatest increase in weight was that of the calves that had the lowest weight at calving, the SMZ value being 0.910 kg at 7 days and 0.610 kg at 30 days respectively days, with insignificant differences between samples ($p < 0.05$, CI = 0.95). This highlights the fact that the calves that had the lowest weights at calving had a higher rate of development under the influence of factors other than the one considered, feeding being the most important.

Table 4. Statistical estimators for DWG (kg) depending on season

Season	Traits	n	$\overline{X}$	$\pm s_{\bar{x}}$	s	V%	Significance
Summer	DWG7	58	0.8	0.062	0.471	59.158	n.s.
	DWG30	63	0.55	0.017	0.131	24.032	*s
Autumn	DWG7	32	0.84	0.076	0.429	51.27	n.s.
	DWG30	38	0.63	0.025	0.151	23.984	n.s.
Winter	DWG7	14	0.71	0.085	0.317	44.738	n.s
	DWG30	17	0.67	0.069	0.284	42.302	*s
Spring	DWG7	28	0.68	0.059	0.315	46.254	n.s.
	DWG30	36	0.53	0.031	0.186	34.926	**s.

*s - significant ($p < 0.01$); **s - significant ($p < 0.05$)

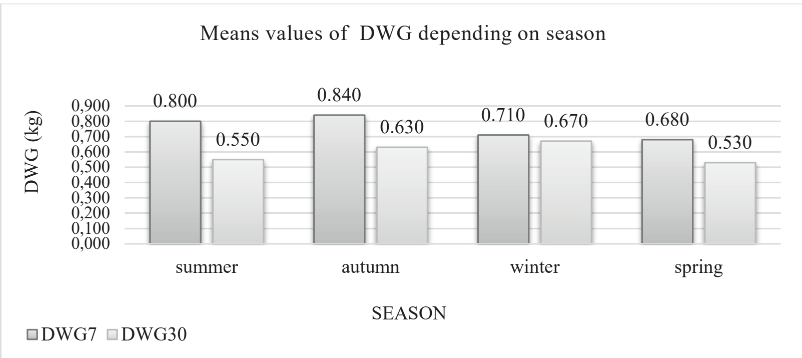

Figure 4. Values of mean for DWG depending on calves` birth weight

Regarding the DWG means according to the calving season, the highest average value for the end of the colostrum administration period is 0.840 kg/day and was recorded in the case of calves calved in autumn and the highest average of DWG30 is in the case of calves calved in winter, with a weight gain of 0.670 kg/day (Table 4 and Figure 4). Values of the coefficient of variation (V%) are over 24% which means that per season, the variability of the character is high. Distinctly significant differences were found between the mean DWG30 in favor of calves calved in winter compared to those calved in spring and summer, the difference between means values being 0.140 kg ($p < 0.01$, CI = 0.95) and 0.120 kg ($p < 0.05$, CI = 0.95). Percentage, spring and summer calved calves had average DWG30 with 21% and respectively 18% lower than those calved in the winter months.

CONCLUSIONS

The daily weight gain of calves is influenced by many factors. In this study, the aim was to highlight the influence of the age at first calving and the parity of the cow, the weight at calving and the season of calving on the DWG of the calves. From the values of the statistical estimators obtained after processing the DWG data, it can be concluded:
- the highest value of the DWG7 were 0.970 kg/day and was recorded for the calves of cows in their second calving and of DWG30 in the case of calves calved in winter which had a weight gain of 0.670 kg/day. The colostrum of multiparous cows has a richer content of antibodies, which is reflected in the health status of the calves;
- the average values of DWG7 are superior to those of DWG30 in all cases analyzed, the rich content of colostrum in nutrients favoring higher gains during this period;
- the values of the coefficient of variability have values higher than 20% in all cases considered, which means that the batch of calves analyzed has a high degree of heterogeneity in terms of weight gain;
- regarding the values of means for DWG30 in calves calved in winter compared to those calved in spring and summer, the differences are 0.140 kg and respectively 0.120 kg in favor of those born in winter, with 21% and 18% higher averages values compared to calving in spring and summer;
- significant differences for DWG7 means were recorded between calves obtained from second-parous cows compared to those from third-parous cows, with the former having an average weight gain of 0.300 kg/day, which means that parity of the cows may be a factor of influence for weight gain on the colostrum period;
- in the case of the batche of calves analyzed, the age at the first calving of the cows and the birth weight of the calves do not influence the weight gain of those up to 7 and 30 days, the differences between the DWG averages being insignificant;
- the weight gain of the calves for the considered periods can be optimized by modulating environmental factors other than those analyzed and by appropriate managerial measures (administration of colostrum, the amount of milk provided as feed etc.).

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THE IMPACT OF INCREASED SOMATIC CELL COUNT ON COW MILK ACIDITY AND LACTOSE CONTENT

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Abstract

Milk quality is increasingly important for producers and consumers as it relates to processing, production, and price. The aim of the present study was to determine the relationship between somatic cells count, lactose content, and acidity of cow milk. A total number of 100 milk samples, from cows with mastitis were processed and analyzed. Increasing acidity and decreasing lactose content were correlated with an increased number of somatic cells in the collected milk samples. The lactose ratio decreased as the number of somatic cells in the milk increased, thus leading to an increase in the titratable acidity of the milk. It can be concluded that a higher number of somatic cells adversely affects milk quality and subsequently processing capacity. Lactose content and titratable acidity can be used as indicators as complimentary to monitor udder health and for early diagnosis of subclinical mastitis in milk cows.

Key words*: acidity, lactose, milk cow, number of somatic cells.*

INTRODUCTION

The quality of cow milk is a critical aspect of dairy production, with somatic cell count (SCC) being a key indicator of udder health and milk quality. Elevated SCC is associated with inflammatory processes in cows and reflects the udder health status and raw milk quality. In the case of clinical mastitis, it causes visibly abnormal milk and an alteration of the udder (Granaci et al., 2023; Schukken et al., 2003).
Subclinical mastitis, on the other hand, does not show any symptoms and can only be evidentiated by counting the somatic cells of a single cow or by analysing microbiological cultures (Ashraf et al., 2020; Hameed et al., 2007). Season, breed, age, condition of the mammary glands, lactation stage, and nutritional management are some variables that affect milk composition (Hristev et al., 2022).
Previous research has shown that the number of somatic cells, as well as the amount of protein and lactose in milk, have a significant impact on milk production (Chen et al., 2021; Pegolo et al., 2021; Rearte et al., 2022). According to Beni et al. (2018), physical damage to the milk-producing epithelial cells is the main cause of the decrease in milk output that occurs along with an increase in milk SCC. Damage to alveolar epithelial cells has also been proposed as a possible explanation for the lactose decrease. Because lactose plays an important role in maintaining milk's osmotic pressure, a decrease in its amount results in a considerable reduction in milk output. Furthermore, sodium chloride ions are transported from the circulation into milk to maintain the osmotic balance, boosting their overall content to an excessively high level (Alhussien et al., 2018; Neculai-Văleanu et al., 2022). Mastitis affects milk's composition, yield, and physical-chemical properties (Cunha et al., 2008). These modifications are linked to changes in the enzymatic activity of somatic cells or microorganisms in the infected mammary gland (Benić et al., 2018; Reis et al., 2013).
Sub-clinically infected cows have somatic cell counts greater than 2.0×10^5 cells/mL (Miglior et al., 2007). Because mastitis infection alters the mammary glands' homeostasis, a drop in the amount of lactose in milk has been linked to an increase in somatic cells. According to Berglund et al. (2007), there is a correlation between a decrease in lactose levels (from 4.86 to 4.69%) and an increase in somatic cell count (from 3.1×10^4 to 4.5×10^5 somatic cells/mL) (Antanaitis et al., 2021).
Researchers have also noted that milk is the primary source of lactose utilized in industry and that lactose is widely employed in the food

and pharmaceutical industries (Ferrari et al., 2004). Lactose is a disaccharide composed primarily of glucose and galactose molecules, accounting for about 40% of the total solids and 50% of the fat-free solids. The health of the udder, the cow's energy balance, and her metabolism all have an impact on the synthesis and concentration of lactose in milk (Antanaitis et al., 2021; Costa et al., 2019; Reis et al., 2013). Additionally, high SCC levels have been linked to decreased milk yield and altered milk components, including lactose. Furthermore, an increase in SCC has been associated with changes in milk acidity, as reflected by alterations in milk composition, such as increased NaCl concentration. These findings underscore the importance of understanding the impact of increased SCC on milk acidity and lactose content (Silva et al., 2018). Because of the lactic acid that bacteria produce during fermentation, cow milk has a mild acidity. Increased SCC has had a mixed effect on milk acidity, according to studies. Some studies suggest a modest rise in acidity with increased SCC, whereas others show no significant difference.

This inconsistency is most likely caused by a combination of factors, including the specific bacteria that cause mastitis, the degree of the inflammation, and individual cow variances (Navarro et al., 2021). The suggested ways that SCC is connected to acidity and lactose are complicated. They involve how inflammatory processes, enzyme activity, and the makeup of bacteria interact with each other. Understanding these mechanisms is crucial for developing mitigation strategies to minimize quality deterioration and the potential health risks associated with elevated SCC milk.

Elevated SCC might lead to lactose degradation by enzymes released from somatic cells or bacteria, resulting in lower lactose levels. This decrease can pose challenges for lactose-intolerant consumers and affect cheesemaking processes that rely on lactose fermentation.

The lactation stage and genetic factors have an impact on the fatty acid composition of milk, which can further alter the acidity and lactose content of the milk (Ben Fraj et al., 2023; Bobe et al., 2007). Additionally, the presence of particular fatty acids as well as other ingredients like minerals and amino acids can affect milk's overall nutritional value and acidity (Li et al., 2011). Although the detrimental effects of high SCC on milk composition-such as protein content and yield-have long been known, the impact on acidity and lactose concentration has received less attention. To close this gap, our study looked into possible connections between changes in these important milk components and elevated SCC.

The dry and wet seasons have a substantial impact on milk quality while also influencing the physicochemical properties of milk (Reis et al., 2013). This study investigates how increasing somatic cell count impacts lactose content and titratable acidity in milk samples from cows with mastitis, regardless of harvest season (warm or cold).

MATERIALS AND METHODS

Experimental Design

Samples were collected from Holstein dairy cows in a farm from north-eastern Romanian, during cold and warm seasons. Animals with high milk conductivity were identified using the loggings from AfiMilk system. The AfiMilk program is the most advanced software in the world for dairy farm management, and we can say that diagnosing subclinical mastitis is considerably easier when the cows' dynamic activity is correlated with the amount of produced milk, and electrical conductivity.

Before the collection of the milk samples, the udder and the milkers' hands were carefully cleansed with soap and water and disinfected with 70 percent ethyl alcohol. The first jets of milk were discarded, and then about 50 ml of milk were collected in sterile containers. The collected samples were analysed, the number of somatic cells, the lactose content, and the titrable acidity were assessed. The collected samples were transported under optimal temperature conditions to the analysis laboratory where to evaluate the number of somatic cells and the lactose content, they were heated in a water bath at 37^{o}C, and for the determination of titratable acidity, the samples were heated in a water bath at temperature of 22^{o} C.

Evaluation of the number of somatic cells in milk

There are several types of methods and equipment available for counting somatic cells in milk, including the direct microscopic cell count, which is used as a high-accuracy test. A quick technique to evaluate the number of somatic cells is SomaScope LFC 600/300.

Figure 1. Analyzer SomaScope LFC 600/300 (Original Image, Laboratory of Nutrition, Quality and Food Safety, R & D Station for Cattle Breeding Dancu, Iasi)

The automated Analyzer SomaScope LFC 600/300 was used to determine somatic cell count (SCC), a technique that offers the advantages of reducing analysis time (200 milk samples/hour), costs, and reagent consumption. The collected milk samples were heated in a water bath at 37ºC, maintained for approximately 30 minutes, stirred, and then analysed (Ariton et al., 2022). To obtain results, we ran a controlled experiment with a group of cows with varied SCC levels (> 4.0 x 10^5 cells/mL). Milk samples were collected in different seasons and tested for the number of somatic cells, lactose content, and level of acidity using standard methods.

Evaluation of the lactose content in milk

The automated analyzer - FTIR Lacto Scope 600/300 was used to determine Lactose content from samples collected. The solutions used for the analysis were: 0.01% Triton solution and 0.4% Decon solution. With this analyser, can determine the main constituents of milk (fat, protein, lactose, casein, urea).

Figure 2. Analyzer - FTIR LactoScope 600/300 (Original Image, Laboratory of Nutrition, Quality and Food Safety, R & D Station for Cattle Breeding Dancu, Iasi)

Evaluation titrable acidity of milk

The acidity of the milk is determined by an acid reaction involving free acids and salts, and it is an indicator of its freshness. Freshly milked milk has a somewhat acidic reaction, but it becomes more acidic with time due to microbial fermentation of lactose and its transformation into lactic acid. The acidity of the milk sample prepared for analysis is neutralized by titration with 0.1 N sodium hydroxide solution and phenolphthalein as an indicator (1%). Milk acidity is measured quantitatively using the titration method (standardized method) and expressed in Thörner degrees (ºT).

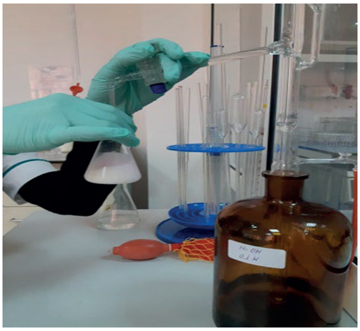

Figure 3. Determination of milk acidity (Original Image, Laboratory of Nutrition, Quality and Food Safety, R & D Station for Cattle Breeding Dancu, Iasi)

Milk acidity, in Thörner degrees, is calculated with the formula:

$$\text{Acidity } (°T) = 10 \times V$$

where:

V - is the volume of the 0.1 N sodium hydroxide solution used in the titration, in ml;

10 - is the volume of the product used, in ml.

As a result, the arithmetic means of three parallel determinations that meet the repeatability condition, values that fall within the range of 15-19 °T, is taken.

Data Analysis and Statistics

Statistical analysis was carried out to assess the relationship between milk parameters and the influence of season. Data on lactose content, acidity titrable, and SCCs were analyzed by one-way analysis processing done in Microsoft Excel, using ANOVA variance analysis test of variance (ANOVA). Pearson's correlation coefficients (r) were computed between total

SCC, milk lactose content and acidity using data from cold and warm season.

RESULTS AND DISCUSSIONS

The purpose of this study was to examine the relationship between somatic cell count (SCC), lactose content, and titrable acidity in both cold and warm seasons. Figure 4 shows the Dynamics of the tested parameters (SCC, Lactose and acidity) according to the collection season of milk samples from cows identified with an increased number of somatic cells (> 400,000 cells/mL).

The comparative analysis of the dynamics of the evaluated parameters according to the sampling season showed that the differences between the values that characterized the two seasons were distinctly significant for the number of somatic cells.

The data were processed statistically to determine the main statistical estimators (mean, variation, standard deviation, standard error of the mean, and coefficient of deviation).

The differences between the averages of the same parameters in the two seasons were calculated and expressed as relative deviations. The ANOVA test was also used to express the degree of significance of the differences between the values of the tested parameters and the seasons (Table 1).

A significant difference was observed between cold and warm seasons considering the SCC, lactose content, and titrable acidity. Moreover, a negative correlation (r = 0.731) was observed between lactose and SCC (Table 2).

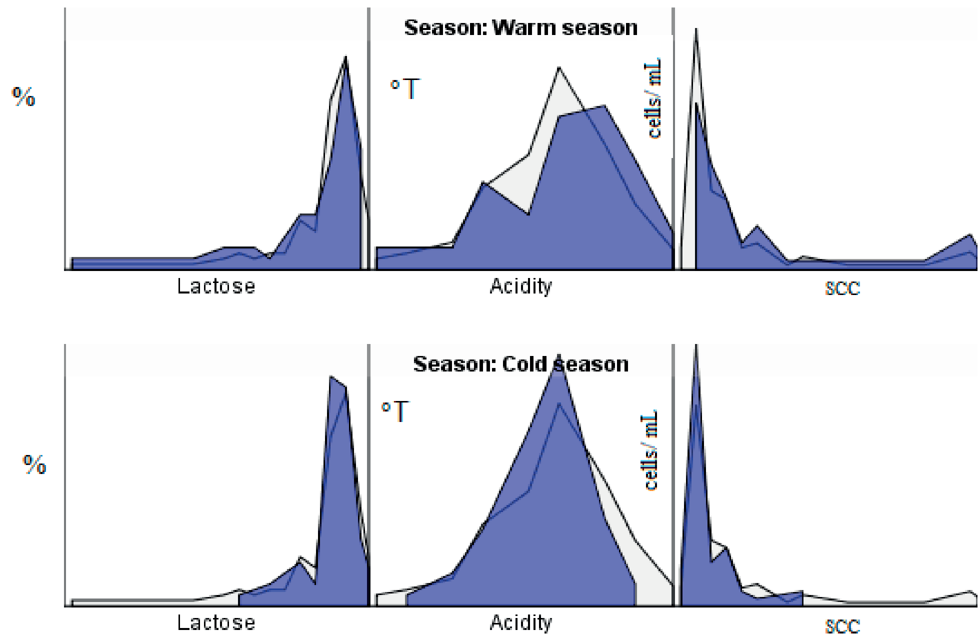

Figure 4. Dynamics of the tested parameters (SCC, Lactose and acidity) according to the collection season of milk samples from cows identified with an increased number of somatic cells

Table 1. Analytical statistical analysis - milk parameters and season dynamic

	Warm season			Cold season			
	Mean ± SD	**Minimum**	**Maximum**	**Mean ± SD**	**Minimum**	**Maximum**	**ANOVA**
Lactose	4.18±.87	0.09	4.92	4.42±0.40	2.94	5.02	p > 0.05
Acidity	18.93±1.89	14.00	22.00	18.48±1.21	15.00	21.00	p > 0.05
SCS	2350433.33± 2476698.39[a]	54900000	8534000.00	908616.67± 695928.87[b]	95000	3710000.00	p < 0.001***

Table 2. Pearson correlations

	Lactose	Acidity	SCC
Lactose	1	-.274**	-.731**
Acidity		1	0.429**
SCC			1

**. Correlation is significant at the 0.01 level (2-tailed)

While the direct effect of increased SCC on cow milk acidity and lactose content may be small, it is nonetheless an important signal of future udder health issues and overall milk quality. Alessio et al. (2016) have indicated that lactose content in milk is influenced by SCC levels, parity, and seasonality, with no significant relationship to breed, milk yield, fat content, or protein levels. Furthermore, environmental factors like temperature and humidity can impact milk composition, with higher temperatures leading to decreased protein, fat, and lactose content, as well as increased microbial counts and SCC (Toghdory et al., 2022).

Intramammary infections can increase SCC and decrease protein, lactose, and fat content in milk (Paape et al., 2001; Vasil et al., 2016). Studies have reported conflicting results. Some suggest a potential increase in milk acidity with higher SCC, potentially due to the release of inflammatory mediators. However, others show no significant change. In our study, acidity was directly influenced by the increase in SCC.

Increased SCC might lead to a slight increase in milk acidity due to the influx of immune cells and enzymes from the inflammatory response. Regarding the lactose content, our findings are consistent with the ones from previous studies which report a decrease in lactose content associated with higher SCC, potentially linked to altered milk synthesis or enzymatic activity.

Maintaining healthy cow populations with low SCC levels is critical for producing high-quality, safe, and nutritional milk for consumers. Understanding the influence of SCC on milk acidity and lactose content is crucial for various stakeholders in the dairy industry. For farmers, this knowledge can help identify cows with potential udder health issues and implement appropriate management strategies.

For processors, information on acidity and lactose content is essential for optimizing processing methods and product quality. Additionally, consumers benefit from understanding how milk quality can be affected by factors like SCC.

CONCLUSIONS

This study provides valuable insights into the relationship between SCC and milk composition, specifically focusing on lactose and acidity. High somatic cell counts (> 4.0 x 10^5 cells per mL) suggest bacterial infection, as evidenced by a strong negative connection between this parameter and lactose concentration. Somatic cell counts correlate negatively with lactose and acidity levels.

Lactose content and titratable acidity can be used as indicators to monitor udder health and for early diagnosis of mastitis in milk cows.

Thus, it is regularly recommended to revise the management practices on the farm and evaluate the basic parameters of the milk.

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THE BIOLOGICAL ROLE OF SHEEP AND COW MILK PROTEINS

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Abstract

The study of milk proteins from different animal species is essential for understanding their impact on human health and their potential in the food industry. This article examines the biological role of proteins found in sheep and cow milk, highlighting their unique contributions to human nutrition and disease prevention. Through a comparative analysis, we explore the specific protein profiles of these two types of milk, including amino acid composition, biological activities, and digestibility. By employing a methodology that integrates in vitro studies, clinical trials, and systematic literature reviews, we assessed the impact of these proteins on bone health, muscle development, and immune system regulation. Our findings reveal that, although sharing some essential nutritional properties, sheep and cow milk proteins exhibit marked differences in terms of immunomodulatory capabilities and the prevention of chronic diseases. Specifically, certain sheep milk proteins have shown superior potential in promoting gastrointestinal health and preventing inflammation. This article concludes on the importance of diversifying protein sources in the diet to fully leverage their health benefits, highlighting the potentially superior role of sheep milk in certain nutritional and therapeutic contexts. Our discoveries open new avenues for exploiting the unique properties of sheep and cow milk proteins, both for nutritional science and industrial applications.

Key words*: milk production, protein, cow, sheep, biological role.*

INTRODUCTION

The protein in the Milk illustrates a significant source of nutrition because of the high biological values and the presence of amino acids essentials. Milk proteins are also the source of different dairy products because of the important techno-functional and biological properties of the proteins. With respect to cow milk, it is depicted as the heterogenous mixture of the proteins having different physio-chemical and structural properties (Petrova et al., 2022). Milks are obtained from the mammalian specie while cow milk protein is categorized in accordance t the solubility in two different fractions including caseins that are insoluble in acidic conditions and the whey proteins that are soluble proteins (Zenker et al., 2020). With respect to the biological value, HBM is approximately 9 gram of the protein per litre, thus, cow milk comprises of 32 gram of protein per litre (Antunes et al., 2022). In the cow milk, there is a ratio of casein fraction that is around 76 to 86% while the fraction of whey protein is around 14 to 24 percent in the total protein content of the cow milk. In addition, there are variations in the content and ratio of the protein subclass in the cow milk composition (Dhesi et al., 2020).

It has been denoted that the consumption of cow milk is by billions of people globally and has been recognized as the overall food. The ratio protein in the cow milk aims to provide both micro and macro nutrients that are important to the development and growth of the human body. The protein is depicted to be of high biological value that aims to represents all important amino acids and have high level of digestion (Giannetti et al., 2021). Despite the nutritional composition, the role of milk products in the human nutrition is high and depicts that it plays a protective and essential role to fight with the chronic disease (Zenker et al., 2020). Another study denoted in the background that cow milk biological protein has major health consequences including the allergy and lactose intolerance while, the choices of people lifestyle such as vegetarianism and veganism, the demand of cow milk alternative have increased and now people are using plant-based milk which have increased the varieties of the plant-based milk (Petrova et al., 2022). People usually considers the plant-based milk as the alternative to the cow milk. The biological role of milk proteins has now been illustrated as negative due to having high ratio of cholesterol and fatty acids and composition of lipid fraction (Auntunes et al., 2022) With regards to the sheep milk, it exerts a ratio of biological ratios which influence metabolic actions and resistance to disease (Nudda et al., 2020). The sheep milk comprises of high ratio of casein, conjugated linoleic acid isomers, whey protein that aims to stimulates the immune systems and comprise of the properties of antidiabetic, anti-obesity and anti-cancer. The sheep milk has been denoted as to have an excellent resource of group B vitamins and protein that assures the good functioning of the nervous system (Nudda et al., 2020). The milk of sheep holds major biological value as it contains proline rich polypeptide that partially reverse the neurodegenerative change and also have an immunoregulatory and properties of pro-cognition (Wang et al., 2022). The biological property of CLA isomers in the sheep milk helps in minimizing the oxidative level of stress and mitrochondrial dysfunction in the brain that helps in reducing neurodegenerative disease including the Alzheimer disease (Lajnaf et al., 2023). The milk of sheep has been denoted as the inhibitory activity that works to reduce the angiotensin and coverts the enzyme that is crucial in the prevention of any infection (Lajnaf et al., 2023). The biological role of sheep milk is significant in lowering down the inflammatory biomarker and also minimize the atherosclerosis development in the human body (Lajnaf et al., 2023) Presently, the lifestyle has become sedentary and there has been lack of physical activities with an insufficient contribution of diet which creates rapid disease development. In the contemporary era, the customer awareness of food depicts that people wants highly biologically active ingredients that could be able to enhance the health thus the functional food plays a significant role in the health of human (Flis & Molik, 2021). With respect to the sheep milk and the products, it is a major source of calcium, fatty acids, iron, magnesium and phosphorus. The study by Li et al. (2022) denoted that the sheep milk protein biological value represents that it is fermented and fermented quality always imprints a health promotional effect specifically on the civilization disease and hearts disease. The consumption of sheep kefir and yogurt minimize the obesity and the risk of metabolic syndrome which reduces the type 2 diabetes (Roy et al., 2021). The consumption of sheep milk comprises of biologically active ingredients, antioxidant ingredients and immunomodulatory substance. There is a high ratio of content of valuable substance in the milk proteins of sheep and has been prove to provide major benefits to human health (Roy et al., 2021).

MATERIALS AND METHODS

For this study, the researcher used qualitative design to review the findings and to conclude the final thoughts. The rationale behind using qualitative research design is that this kind of design helps in exploring the objectives and to provide a deep understanding on the insights of the real-world facts. For this study, the researcher has not used the numerical data or have intervened with the statistics but used qualitative research to obtain the hypotheses. The qualitative study design helps the researcher to obtain the experiences of the researchers, their behaviour and perception regarding the concerned topic while, it helped in responding the whys and hows instead of how much and how many (Tomaszewski et al., 2020).

In order to analyse the biological role of sheep and cow milk proteins, the literature analysis has been done. The analysis of literature comprises of two different forms that are within the study literature and between the study literature analysis (Paul & Criado, 2020). Both the analysis of literature is important. This study used within study literature evaluation that includes to analyse the contents from the past journal articles and researches. The study has analysed every component related to the research objectives from different past research papers including their section of literature review, theories, conceptual frameworks, discussion and results (Paul & Criado, 2020). By analyzing the literature and past research papers, the common data and information have been obtained from the empirical works to attain the findings and to sum up the conclusion.

Initially, the researcher started the preparation stage and prepared the review and research structure by evaluating about the specific research objectives and questions to be addressed. After searching all the relevant literature, the researcher analysed that there is a need to obtain the articles information that should be up to date and must be related to the topic. The recent articles have been assessed to obtain the biological significance of the sheep and cow milk protein and its effectiveness on human health.
The searching was done by using appropriate key words and articles and research were analysed from the scientific database. Some of the scientific databases that were used includes PubMed, Scopus, Science Direct and Web of Science. This analysis included proper inclusion for every publication used in the study thus, specific principles were being formed as the inclusion criteria in this study. In the data collection, the most up to date scientific articles were used from the year 2019-2023. Thus, major prominence was placed on the research papers which were published within the last five years which increases the viability of this study.

RESULTS AND DISCUSSIONS

The study denoted about the components of the sheep milk proteins signifying that the significant endogenous amino acid in the sheep milk protein is called as proline that plays a major role in the synthesizing of the polyamines and arginine and also activates the MTOR cells which signals to initiate the process of the synthesis of proteins, especially collagen. Both of them are found in the sheep milk.

Table 1. The composition of sheep milk proteins (Flis & Molik, 2021)

Amino acid	In g/100 g of sheep milk	In g/100 g of Casei
Tryptophan	0.084	1.3
Threonine	0.268	3.6
Isoleucine	0.338	5.1
Leucine	0.587	9
Lysine	0.513	7.3
Methionine	0.155	2.1
Cysteine	0.035	0.8
Phenylalanine	0.284	5.2
Tyrosine	0.281	5.6
Valine	0.448	6.7
Arginine	0.198	3.3
Histidine	0.167	3.3
Alanine	0.269	3.2
Aspartic acid	0.328	7.7
Glutamic acid	1.019	21.1
Glycine	0.041	1.7
Proline		10
Serine	0.492	5

With respect to importance of each amino acids for human health, here are following points of some of them.
Histidine amino acid is important for the repair and growth od tissues, protection of nerves and helps in the histamine production that is essential for boosting human immune system
The valine, leucine and Isoleucine provides strength to muscle and provides energy production during the exercise by regulating the sugar levels in blood.
Lysine is helpful to provide protein, formation of collagen, absorption of calcium and carnitine production that is included in energy production and metabolism of fats
Methionine supports in the detoxification process and contains compounds of sulphur that is important for human health (Lopez & Mohiuddin, 2023).

Table 2. Essential amino acids in cow milk (Kyselov et al., 2022)

Essential amino acids	Cow milk
Tryptophan	0.046
Threonine	0.149
Isoleucine	0.199
Leucine	0.322
Lysine	0.261
Methionine	0.083
Cystine	0.03
Tyrosine	0.159
Valine	0.22

The table indicates that cow milk is a rich source of essential amino acids, which are the building blocks of proteins necessary for numerous bodily functions. Tryptophan, although present in the smallest amount, plays a key role in the synthesis of serotonin, contributing to mood and sleep regulation. Threonine supports protein synthesis and immune function, while isoleucine, leucine, and valine - all branched-chain amino acids - are abundant and critical for muscle repair and energy. Leucine, in particular, stands out for its role in muscle protein synthesis. Lysine is vital for tissue repair and metabolism, methionine for detoxification processes, cystine for structural protein formation, and tyrosine for neurotransmitter production. The presence of these amino acids in adequate amounts confirms the nutritional value of cow milk, especially as a source of complete protein in the diet.

Table 3. The nutritional composition of cow and sheep milk (Zgheib & Zara, n.d)

Parameter g/100 g	Cow milk	Sheep milk
Moisture	87.9	82.9
Fat	3.7	6.1
Ash	0.7	0.9
Lactose	4.7	4.8
Protein	3.4	5.5
Casein	3	4.7

This table compares the compositional differences between cow milk and sheep milk based on various nutritional parameters per 100 grams:
Moisture: Sheep milk has less moisture content (82.9 g/100 g) compared to cow milk (87.9 g/100 g), which implies that sheep milk is denser and potentially more concentrated in other constituents.
Fat: The fat content in sheep milk (6.1 g/100 g) is significantly higher than in cow milk (3.7 g/100 g). This higher fat content can contribute to a creamier texture and richer flavor in sheep milk and its derived dairy products. It can also mean higher energy content per unit volume.
Ash: The ash content, which is a proxy for total mineral content, is slightly higher in sheep milk (0.9 g/100 g) compared to cow milk (0.7 g/100 g). This suggests that sheep milk may offer more minerals, which are essential for various body functions.
Lactose: Both types of milk have nearly the same lactose content, with sheep milk having a marginally higher amount (4.8 g/100 g) than cow milk (4.7 g/100 g). Lactose is the sugar found in milk and is an important carbohydrate source.
Protein: Sheep milk has a considerably higher protein content (5.5 g/100 g) than cow milk (3.4 g/100 g). Proteins are crucial for body repair and growth, and sheep milk's higher protein content could make it a more nutritious option.
Casein: Casein is the main group of proteins in milk and forms the basis of cheese. Sheep milk contains more casein (4.7 g/100 g) than cow milk (3 g/100 g). Higher casein content can lead to increased cheese yield from sheep milk and might affect the texture and flavor of the cheese. In summary, sheep milk appears to be richer in several key nutrients compared to cow milk. This can make it a valuable food source, especially in regions where sheep are more prevalent than cows. The higher fat and protein contents also suggest that sheep milk may have a more pronounced taste and could be more satiating. However, these differences also mean that sheep milk might not be as suitable for individuals with specific dietary restrictions, such as those requiring lower fat intake. The nutritional richness of sheep milk, especially in terms of proteins and minerals, highlights its potential benefits and uses in various culinary and food processing applications.

Components in the cow milk

The past study by Lajnaf et al (2022) denoted the components in the cow milk proteins signifying Caseins as the important component that are the phosphoproteins that illustrates the sufficient fraction of protein in the milk and represents 80 percent of the total protein in the milk. It usually comprises of four proteins which differs with respect to the content of concentration, amino acids, phosphorus and molecular weight. In the protein, α and β caseins are denoted as the most sensitive caseins and involves in the precipitation of the concentration of calcium (Lajnaf et al., 2022).

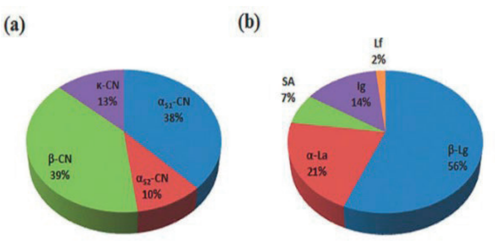

Figure 1. Proportions of the different caseins (a) and whey proteins (b) in cow's milk (abbreviations: β-CN: β-casein; αs1-CN: αs1-casein; αs2-CN: αs2-casein; κ-CN: κ-casein, β-Lg: β-lactoglobulin; α-La: α-lactalbumin; SA: serum albumin; Ig: immunoglobulins; Lf: lactoferrin (Lajnaf et al., 2022)

The Figure 1 denotes that the cow milk also contains the soluble protein fraction and the whey proteins that are the most significant component and are in the high fraction in the milk having the percentage of 20% to 25%. The composition of the whey protein in the cow milk relies on the species of mammalian. Thus, the table denotes that the ratio of lactoglobulin is 56 while, α lactalbumin is 21 percent and bovine serum albumin is 7 percent (Lajnaf et al., 2022)

Table 4. Characterization and Allergenic Profile of Milk Proteins in Cow's Milk (Lajnaf et al., 2022)

Specification	Proteins	Allergen name	Molecular mass	PI	Relative amount	Amino acid residus	Allergenic activity of
Caseins 80% of total protein	Cas ein	Bosd9	22.9	4.46	38%	199	57%
	Cas ein	Bosd10	22.4	4.78	10%	207	
	Cas ein	Bosd11	23.5	4.49	39%	209	
	Cas ein	Bosd12	18.9	3.97	13%	169	
Whey proteins (20-25%)	Lg	Bosd5	18.28	5.2	56%	162	66%
	La	Bosd4	14.18	4.65	21%	123	18%
	BSA	Bosd6	66.4	4.7	7%	583	
	Lf	Bosd lactofer rin	76.1	8.18	2%	689	
	Ig	Bosd7	15.-800	5.5-7.5	14%	240-250	

The table presents an insightful breakdown of the main protein allergens found in cow's milk, detailing caseins and whey proteins. Caseins, which make up approximately 80% of total milk protein, are divided into four subtypes: Bosd9, Bosd10, Bosd11, and Bosd12, with molecular masses ranging from 18.9 to 24.4 kDa and pI values between 3.97 and 4.78. The relative amounts of these caseins vary, with Bosd11 constituting the largest proportion at 39%, while Bosd12 is the least at 13%. The allergenic activity, where noted, shows that 57% of patients exhibit a reaction to Bosd9, a significant figure suggesting its high allergenic potential.

Whey proteins, which account for 20-25% of milk proteins, include β-lactoglobulin (Bosd5), α-lactalbumin (Bosd4), bovine serum albumin (BSA, Bosd6), lactoferrin (Bosd lactoferrin), and immunoglobulins (Ig, Bosd7). These proteins have molecular masses that range broadly from 14.18 to 800 kDa and show varied allergenic activity, with β-lactoglobulin affecting 66% of patients, highlighting its prominence as a milk allergen.

Notably, α-lactalbumin has a lower incidence of allergenic activity (18%), suggesting it might be less problematic for people with milk allergies. However, lactoferrin and immunoglobulins, despite their lower relative amounts in milk, have significant molecular sizes and varying pI values, which could influence their allergenicity.

This table is a valuable resource for understanding the allergenic components of cow's milk. It also emphasizes the complexity of milk allergies, as various proteins contribute differently to allergenic responses. The data could be pivotal for developing hypoallergenic milk variants and for healthcare professionals managing patients with milk allergies.

Biological role of cow milk proteins in functioning the human body

It has been denoted in the literature that the cow milk proteins are highly efficient in the functioning of human body as the milk is rich source of different proteins and each component in the milk proteins plays an effective biological role in the human body (Ribes-Konincks et al., 2023). One of the past studies identifying the efficiency of cow milk proteins signifies that the primary proteins in the cow milk are whey proteins and the casein. With respect to the biological role of Casein, the past literature emphasizes that Casein helps in making up around 80 percent of the total content of protein in the cow milk and exists in the form of micelles that are the tiny and little particles (Cronin et al., 2023). The cow milk contains Casein hence it is digested slowly by the human beings and provides a sustain releasing of the amino acids in the bloodstream of the humans and keeps the human body full for long period of time (Cronin et al., 2023). The study also emphasized that Casein plays an important role in the transport of the phosphorus and calcium in the body that helps in strengthening the metabolism of the human (Cronin et al., 2023). In the cow milk, there are also whey proteins included that are depicted as the high-quality proteins and are important for the human body functioning (Lajnaf et al., 2023). The whey proteins in the cow milk also supports in the fast digestion in the human body and leads to increase the amino acid levels in the bloodstream which makes it a good source of protein for the recovery of any disease (Balivo et al., 2023). The study denoted that the wo milk proteins comprise of immunoglobulins which significantly contributes in the function of the immune system to safeguard the body from germs (Lajnaf et al., 2023). The lactoglobulins and lactalbumin are also denoted as the proteins that plays an essential role in the transportation of the important nutrients such as minerals and vitamins in the human body while the component of lactoferrin helps to bind with iron and transports absorption that contributes in defending the body from all kind of infections (Lajnaf et al., 2023)

Biological role of sheep milk proteins in functioning the human body

The literature from the past study Yang et al (2023) denoted that the sheep milk protein is highly efficient in the functioning of the human body as it contains anti-cancer, antioxidant and anti-inflammatory impact on the body. The research has proved that the sheep milk proteins have the capability to reduce the oxidative stress level in the body and also regenerates the antivirus to fight with the HIV and hepatitis C (Cunha et al., 2023). The sheep milk proteins are also important in binding the irons and blocks the growth of infection and microorganism in the body. It has the capability to inhibit the cancer cell proliferation and also have the capability to fight with the blood brain barricades (Pan et al., 2023).

Comparison between protein content of cow and sheep milk

The sheep and cow milk differs with respect to their protein content that could have effect on the dietary preference and needs of human. In the cow milk, the typical protein content is around 3.2% to 3.5% while, sheep milk contains high ratio of protein that ranges from 4.5% to 6%. With respect to Casein and whey ratio, cow milk holds high ratio of casein and whey proteins that helps to increase immune system in human while, sheep milk has a balanced proportion of casein and whey protein in comparison to cow milk (Landi et al., 2021). Sheep milk is easy to digest and absorb due to balanced proportion. Both the sheep and cow milk comprise of important amino acids but sheep milk have high concentration of amino acids in comparison to cow milk. In a nutshell, the protein content in sheep milk is high than the cow milk and sheep milk is more beneficial for the people who urged to take proteins more hence, cow milk is preferable for people who looks for moderate protein intake (Landi et al., 2021).

CONCLUSIONS

The study aims to evaluate the biological role of cow and sheep milk proteins for the functioning of human body however, the findings obtained from various literature search depicted that sheep milk is more nutritious in proteins in comparison to the cow milk as sheep milk is different from the cow milk holding more proteins in milk per glass. In addition, sheep milk has high ratio of fats, vitamins, proteins and fats and adds the double level of zinc and calcium in comparison to the milk of cow. The components studied in the findings and discussion part also concluded that the protein in the milk of sheep is more digestive in comparison to the milk of cow as the milk of sheep aims to deliver high ratio of amino acids, leucine, isoleucine and valine and helps in building the blocks of protein in the human body. The study also concludes that the biological role of sheep milk proteins comprises of high ratio of saturated fatty acids and increase the lactose absorption that is also beneficial for those who are intolerant to lactose. The nutritional value of the sheep milk protein is high than the cow milk proteins as it supports the human body to strengthen the immune system and to fight with the disease of heart, epilepsy, gall stones and cystic fibrosis. In a nutshell, the study summarizes that the value and biological role of sheep milk protein is highly effective for the human body functioning.

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CURRENT RESEARCH STATUS ON SOME NUTRITIONAL SOLUTIONS FOR SWINE FEEDING TO IMPROVE PRODUCTIVE PARAMETERS

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Abstract

In the context of the development of livestock industry, pig farming represents an important sector, making it essential to optimize the feeding regime to improve productive parameters. Recent research has focused on identifying and implementing innovative nutritional solutions that support swine performance, with the main objective of increasing resource use efficiency, improving feed conversion, and reducing environmental impact. Alternatives to conventional proteins, such as insect or algae-based proteins, have been investigated, which could reduce dependence on traditional protein sources like soy and lower production costs. That dietary adjustments can lead to increased body weight, improved feed conversion ratio, and reduced pig mortality. Moreover, personalized feeding tailored to the needs of each stage of swine growth has proven effective in maximizing productivity using Precision Nutrition and Data-Driven Feeding. However, achieving a significant impact requires closer collaboration between researchers, farmers, and the feed industry. The large-scale implementation of these promising nutritional solutions necessitates additional efforts in farmer education and ensuring access to advanced technologies and necessary resources.

Key words*: nutritional solutions, productive parameters, swine performance, sustainable development.*

INTRODUCTION

Pig farming is one of the most important branches of animal husbandry, with a significant impact on the national economy and the food market. In recent years, the emphasis on improving the productive parameters of pigs has led to an increasing need for innovative nutritional solutions that optimize the growth and health of these animals.

Proper nutrition plays a crucial role in productive performance, influencing both the growth rate and the quality of the meat produced (Manceron et al., 2014).

Nutrition is one of the most important factors influencing the productive performance of pigs. A balanced and adequate diet contributes to optimizing growth rate, improving feed conversion, and maintaining the overall health of the animals.

In the context of globalized markets and the increasing demand for high-quality pork products, producers are motivated to adopt advanced nutritional solutions that meet these requirements.

This article analyzes the current state of research on nutritional solutions for pigs and their impact on productive parameters.

MATERIALS AND METHODS

This study employed a bibliographic approach to systematically review, synthesize, and analyze relevant literature on the selected research topic. The methodology focused on identifying and evaluating peer-reviewed academic sources, including journal articles, books, conference proceedings, and reports, to gather comprehensive insights on the subject matter.

The bibliographic review followed a structured search protocol to ensure a wide and thorough inclusion of relevant literature. The following electronic databases were utilized for the literature search: PubMed; Scopus; Web of Science; Google Scholar.

The search criteria were further refined by using filters such as publication date (studies published between 2010-2023), language (English), and type of publication (peer-reviewed articles).

RESULTS AND DISCUSSIONS

Research in pig nutrition has evolved significantly, focusing on identifying and implementing solutions that ensure sustainable and efficient growth. This research has been driven by the need to reduce production costs, improve product quality, and minimize negative environmental impact.

Major research directions include the use of feed additives, alternative proteins, and nutritional supplements, as well as optimizing diet composition according to the growth stages of pigs. In the last decade, numerous studies have been conducted to evaluate the effectiveness of these solutions in the specific context of international and national farms.

Feed additives and their impact on productive performance

Feed additives have become a major topic of interest in pig nutrition due to their potential to improve feed digestibility and stimulate growth. The researchers have explored the use of additives such as probiotics, prebiotics, enzymes, and organic acids.

Probiotics and prebiotics have been studied for their ability to modulate the intestinal micro-flora, promoting gut health and improving nutrient absorption. Studies conducted on pig farms have shown that using these additives can reduce the incidence of digestive disorders and improve the feed conversion ratio (Jacela et al., 2010).

Like probiotics in pig feeding, it can be used *Lactobacillus* spp., which is known for their role in lactic acid production. these bacteria help maintain a healthy gut environment by lowering pH and inhibiting harmful pathogens. *Bifidobacterium* spp. promote gut health by competing with pathogens and supporting the development of a balanced gut microbiota. *Saccharomyces cerevisiae* (yeast) can improve feed digestibility and nutrient absorption, while also boosting immune function. *Enterococcus faecium* helps reduce pathogenic bacteria in the gut and can improve intestinal health and nutrient uptake (Méndez-Palacios et al., 2018).

Common prebiotics in pig nutrition are fructooligosaccharides - promote the growth of beneficial bacteria like *Bifidobacteria* and *Lactobacillus*; mannan-oligosaccharides enhances gut health by promoting beneficial bacteria and binding to pathogens, preventing them from adhering to the gut lining; inulin stimulates the growth of beneficial bacteria while improving digestion and nutrient absorption; galactooligosaccharides feed beneficial bacteria and improve gut microbial balance, supporting better digestive health (Shim, 2005; Kiernan et al., 2023).

Enzymes, particularly those that break down plant fibers, have also been evaluated for their effect on the digestibility of fiber-rich feeds. While pigs naturally produce some digestive enzymes (e.g., amylase, protease, lipase), they are often insufficient to fully digest certain components of the diet, especially when it contains plant-based feedstuffs like cereals and legumes. These enzymes help release essential nutrients from feed ingredients, thereby increasing diet efficiency and contributing to faster and more uniform pig growth. Carbohydrases break down complex carbohydrates, which are abundant in plant-based feed ingredients such as cereals, grains, and legumes. Pigs, especially young ones, have limited ability to digest some carbohydrates, particularly the non-starch polysaccharides found in plant cell walls. Xylanase breaks down arabinoxylans into simpler sugars, improving nutrient digestibility and energy extraction. β-Glucanase breaks down β-glucans, which are found in grains like oats and barley, enhancing the digestibility of these feed components. Amylase helps break down starches into sugars, improving the energy availability from grains such as corn and wheat. Cellulase breaks down cellulose, a key component of plant cell walls, although its use is less common due to the difficulty of fully digesting cellulose in pig diets (Durán, 2014; Aranda-Aguirre et al., 2021).

The use of organic acids in pig feeding has gained significant attention due to their beneficial effects on gut health, growth performance, and overall feed efficiency. Organic acids (citric acid, propionic acid, fumaric acid, lactic acid, formic acid, benzoic acid) serve as natural alternatives to antibiotics, especially in post-weaning piglets, by helping to control pathogenic bacteria, improve nutrient absorption, and promote optimal digestive conditions. When supplemented in pig diets, organic acids can improve feed utilization, reduce gastrointestinal disorders, and enhance overall growth performance. They work by lowering the pH of the gastrointestinal tract, creating an environment less favorable for pathogenic bacteria, and more conducive to beneficial microbes and optimal nutrient digestion (Suiryanrayna & Ramana, 2015).

Alternative Proteins: Innovative Solutions for Cost Reduction and Improved Sustainability

Proteins are an essential component of pig diets, with a direct impact on their growth and development. In the context of rising prices for conventional proteins such as soy, researchers have explored alternatives that are both effective and environmentally sustainable (DiGiacomo & Leury, 2019; Lestingi, 2024).

Insect and algae proteins are two of the most promising alternative protein sources that have been investigated. These not only offer a rich nutritional profile but also have a lower environmental impact, requiring fewer resources for production compared to conventional crops.

Insect proteins are considered an excellent source of essential amino acids and have been successfully tested in pig diets, demonstrating an efficiency comparable to traditional proteins. The studies have shown that partially replacing conventional proteins with insect proteins does not compromise pigs' productive performance and may even improve certain aspects of animal health (Lestingi, 2024).

Species like black soldier fly larvae (*Hermetia illucens*), mealworms (*Tenebrio molitor*), and common housefly (*Musca domestica*) are commonly used in animal feed formulations (Wang & Shelomi, 2017; Hong et al., 2020; Hong & Kim, 2022).

Insects contain protein levels comparable to or higher than traditional sources like soy or fishmeal. For instance, black soldier fly larvae have been reported to contain 40-50% protein on a dry matter basis, while mealworms contain approximately 45-50%. These proteins are rich in essential amino acids such as lysine and methionine, which are crucial for pig growth and muscle development (Nowak et al., 2016; Veldkamp, T.; Bosch, 2015).

Insects are also a good source of lipids, ranging from 15-35% depending on the species and life stage. These fats provide essential fatty acids, such as linoleic acid, which contribute to energy balance and overall health in pigs (da-Silva et al., 2024).

Insects provide a wide range of micronutrients. Black soldier fly larvae, for instance, are rich in calcium and phosphorus, which are important for bone development and metabolic functions in pigs. Insects also contain bioactive compounds, such as antimicrobial peptides, that can enhance gut health and immune function in pigs (Wang & Shelomi, 2017).

Pigs fed insect protein often show comparable or even superior growth rates to those fed traditional protein sources. Research indicates that insect meals, especially from black soldier fly larvae, can improve the feed conversion ratio (FCR), meaning pigs require less feed to gain the same amount of weight, thus enhancing feed efficiency. In some studies, FCR improvements of up to 10% have been observed when insects are included in the diet (DiGiacomo & Leury, 2019).

Insects are generally well-digested by pigs, with high protein digestibility rates reported for black soldier fly larvae and mealworm meals. Studies have shown that pigs can efficiently utilize the nutrients in insect-based feeds, with digestibility coefficients similar to those of soy and fishmeal. The acceptability of insect-based feed to pigs has been a subject of research, with most studies reporting that pigs readily consume insect-containing diets without issues of reduced feed intake or rejection (Kar et al., 2021). The flavor and texture of insect meals, combined with their high nutrient density, make them a suitable feed option for swine (DiGiacomo & Leury, 2019).

Insects, particularly their chitin-rich exoskeletons, have been shown to modulate gut microbiota by promoting the growth of beneficial bacteria, such as *Lactobacillus* and *Bifidobacterium*. This can enhance gut health, nutrient absorption, and overall immunity in pigs (Kar et al., 2021).

Insects produce antimicrobial peptides that have been demonstrated to reduce pathogen load in the gut. For example, black soldier fly larvae contain compounds that inhibit the growth of harmful bacteria like *Escherichia coli* and *Salmonella*, thus contributing to improved gut health and reduced infection risk (Yu et al., 2019).

The use of insects in pig nutrition also offers significant environmental benefits, making them a sustainable alternative to traditional feed sources. Insect farming requires far less land and water than conventional feed protein sources like soy or fishmeal. For example, black soldier fly larvae can be produced on food waste, agricultural by-products, or organic waste streams, making them a circular economy solution that reduces the ecological footprint of pig production. Insects emit fewer greenhouse gases during production compared to livestock feed crops such as soy. By integrating insects into pig feed, the overall carbon footprint of pork production can be reduced, aligning with global efforts to mitigate climate change (FAO, 2024; Groeneveld et al., 2021).

Algae have emerged as a promising alternative protein source in animal nutrition, particularly in pig diets, due to their high protein content, rich nutrient profile, and sustainable production. This has attracted considerable attention in both research and the agricultural industry

Algae are another protein source that has gained attention in nutritional research. Algae can be grown on non-arable land and can utilize waste streams (e.g., CO_2 and wastewater), reducing their environmental impact. This makes them an eco-friendly alternative that aligns with the push for more sustainable livestock production systems.

Algae, particularly microalgae (e.g., *Spirulina platensis, Chlorella vulgaris,* and *Schizochytrium*), are known for their high protein content, which can range from 20% to 70%, depending on the species and growing conditions. Algae also contain essential amino acids that are critical for pig growth and development, making them comparable to conventional protein sources like soybean meal and fishmeal (Holman et al., 2013).

In addition to protein, algae are rich in: lipids, particularly omega-3 fatty acids like DHA (docosahexaenoic acid), which can enhance the fatty acid profile of pork; vitamins such as B12, E, and carotenoids like beta-carotene; minerals including iron, calcium, and magnesium (Ribeiro et al., 2021).

Research has shown that algae can promote growth in pigs, particularly weanling pigs. The protein and essential amino acids in algae support muscle development, while the bioactive compounds may enhance feed efficiency (Lugarà et al., 2022).

Algae contain bioactive components like polysaccharides and polyunsaturated fatty acids (PUFAs) that may support gut health and immune function in pigs. Some studies suggest that algae can improve gut microbiota composition, reduce gut inflammation, and increase the pigs' resistance to disease (Kovač et al., 2013).

The inclusion of algae in pig diets has been shown to improve pork quality. Specifically, omega-3-rich algae can enhance the fatty acid profile of pork, resulting in healthier meat with a higher content of beneficial fats (Sánchez-Muros et al., 2014).

While algae have clear benefits, there are several factors to consider when the farmers want to use this forage. The production cost of algae can be higher compared to traditional feed ingredients like soybean meal. However, technological advancements and large-scale production may lower these costs in the future (Austic et al., 2013).

Algae cell walls can be difficult to digest for pigs due to their high fiber content. Processing methods such as cell wall disruption or the use of enzymes can improve digestibility and nutrient availability (Austic et al., 2013).

Some algae species may have a strong odor or taste, which could affect feed intake. Research is ongoing to find ways to enhance the palatability of algae-based feeds (Chisti, 2014).

Research in Romania has begun exploring the potential of algae in pig feed, with preliminary results suggesting an improvement in productive parameters, particularly in meat quality and animal health (Nistor, 2010; Popescu, 2016; Teodorescu et al., 2023).

Precision Nutrition and Data-Driven Feeding

The use of precision nutrition techniques allows farmers to tailor diets to the specific needs of pigs based on factors like age, weight, breed, and health status. Advancements in technology and data analytics provide the tools to optimize feed formulations and reduce waste.

Tailoring feed formulas to different growth phases ensures that pigs receive the precise nutrients they need at each stage, avoiding over- or underfeeding. This reduces feed costs and nutrient excretion. The nutritional needs of pigs vary significantly depending on age, weight, and stage of development, and adjusting the diet to meet these needs can lead to significant improvements in productive performance (Banhazi et al., 2012).

The diet during the weaning period is essential to ensure a smooth transition from maternal milk to solid food. Researchers have shown that a diet rich in energy and proteins, supplemented with enzymes and probiotics, can reduce weaning stress and support rapid and healthy growth (Pop et al., 2006).

The growth and fattening phases require nutrition that supports rapid growth and proper muscle development. Here, the focus is on balancing proteins, carbohydrates, and lipids to optimize feed conversion into muscle mass. Researches have explored the use of cereal-based diets enriched with alternative protein sources and feed additives, demonstrating significant improvements in growth rate and feed efficiency (Simeanu, 2018).

Automated feeders equipped with sensors and artificial intelligence can monitor individual pig feed intake and growth rates in real time (Gaillard et al., 2020). These systems adjust feed formulations dynamically, improving growth performance and feed efficiency.

Continuous monitoring and adjustment are at the core of precision feeding. The systems collect data from various sensors, which is then processed to determine the exact nutrient mix each pig or group needs at any given time. The goal is to maximize growth efficiency while reducing feed wastage and nutrient excretion (Pomar & Remus, 2019).

Systems gather data on feed intake, weight gain, health status, and environmental conditions (e.g., temperature, humidity) to adjust feeding in real time (Flachowsky & Kamphues, 2012; Niemann et al., 2011).

Based on the data, the system can change the ratio of protein, fat, fiber, and micronutrients in the feed to match current nutritional needs (Lovato et al., 2017).

Systems can modify feed delivery not only based on nutritional needs but also on factors such as environmental stress (e.g., heat stress) or health issues (Niemann et al., 2011).

Implementation and practical challenges

Although research shows considerable potential for improving pigs' productive parameters through innovative nutritional solutions, implementing these in practice presents significant challenges. One of the main challenges is educating farmers about the benefits of these solutions and the correct application methods. Additionally, the initial costs of implementing advanced nutritional solutions can be a barrier for many farms, especially small and medium-sized ones.

Another challenge is the limited access to necessary resources, such as alternative proteins or high-quality feed additives, especially in more isolated rural regions. Moreover, closer collaboration between researchers, feed producers, and farmers is needed to ensure the successful large-scale implementation of these solutions.

The future of nutritional research is moving towards developing personalized feeding strategies tailored not only to the growth stages of pigs but also to the specific conditions of each farm. There is also growing interest in nutritional solutions that reduce environmental impact, such as low-carbon diets and the use of local and renewable resources.

CONCLUSIONS

The current state of research on nutritional solutions for pigs shows considerable potential for improving productive parameters. The use of feed additives, alternative proteins, and personalized diets can lead to more efficient growth, improved meat quality, and reduced environmental impact. However, to fully capitalize on these findings, close collaboration between researchers, farmers, and the feed industry is essential, along with continued investment in education and advanced technologies. The large-scale application of these solutions can significantly contribute to the sustainable development of pig sector, offering important economic and ecological benefits.

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RESEARCH ON QUANTITATIVE APTITUDES IN THE DIRECTION OF MEAT PRODUCTION IN LAMBS FROM ȚURCANĂ AND ȚIGAIE BREEDS

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Abstract

This paper aims to quantify the quantitative performances to the production of meat of lambs from the main native sheep breeds, in relation to several influencing factors: breed, type of birth and sex of lambs. To evaluate the degree of body development of the lambs during the birth-weaning period (0-60 days), they were weighed at birth, at 30 days and at weaning (60 days). The study was completed by a phenotypic analysis of the aptitudes for meat production on the live animal carried out by the "Method of points" in the lambs after the completion of the experiment. The best results, in terms of quantitative parameters in meat production (average growth rate, total growth rate, weight at slaughter) were obtained by lambs of the Țigaie breed.

Key words: *breed, body weight, growth, lambs, type of birth.*

INTRODUCTION

Sheep are a species of animals preferred by many breeders due to the multiple particularities materialized through: rusticity, adaptability, the readiness of the species to adapt to a predominantly pastoral exploitation system, the special possibility of efficient utilization of fodder with a high level of cellulose (Călin, 2004).

Also, sheep provide breeders with a wide range of products: milk, meat, hides, skins, hair production, etc.) products that contribute to covering the ever-increasing needs of animal protein, of the population in conditions of economic efficiency.

Meat production, especially the component represented by fattened youth meat, is increasingly requested by consumers, both from our country and especially for export, its quantitative and especially qualitative improvement representing a priority objective for all breeders (Vicovan et al., 2009).

In this sense, one of the priority objectives of this work is represented by the quantification of the main quantitative parameters in meat production (average growth rate, total growth rate, weight at slaughter) in direct correlation with three important influencing factors, respectively breed, type of birth and sex of lambs.

MATERIALS AND METHODS

The biological material studied in this work was represented by the youth flocks of sheep, current year, belonging to two of the most important sheep breeds in our country, the Țurcană breed and the Țigaie breed, raised in a private farm within the Center Development Region, Covasna County. The farm in Covasna county has sheep from the Țigaie breed, the rusty variety, and sheep from the Țurcană breed, the white variety.

Within the farm, a semi-intensive exploitation system is practiced, characterized by the maintenance of sheep on pasture, during the summer period, with the use, in a first stage, of natural pastures, of cultivated and occasional meadows in the vicinity of the farm, following as a period of three months (June, July and August), the sheep should be kept on the foothill and mountain pastures, rented by the owner. During the winter, the maintenance of the sheep is carried out in the shelter, the feeding being based on canned fodder, hay, roughage, silage fodder and concentrate mixtures.

In order to evaluate the degree of body development of the lambs from the two breeds, during the birth - weaning period (60 days) weightings were carried out at birth, at 30 days and after weaning at the age of 60 days, determining the gain of average daily growth and the total weight gain achieved by it, by stages and during the entire tracked period. All the parameters mentioned above were determined separately for lambs, depending on sex, breed and type of birth.

The phenotypic assessment of the aptitudes for meat production in lambs of each breed was carried out on the live animal by the "Point Method" using the "Scoring Table for the assessment of the state of fattening of sheep". Through this method, each valued region is given a grade on the 1-5 grading scale, grade that is amplified by certain coefficients (whose value is directly proportional to the importance of the analyzed region, viewed through the prism of the main production achieved) written in the sheets of scoring. The value of the marks awarded has the following meanings: 5 = perfect; 4 = very good; 3 = good; 2 = acceptable; 1 = bad.

Taking into account the sum given by the total points awarded, the exterior of an examined sheep can be classified as follows:

- Very good exterior ……….86.1-100.0 points;
- Good exterior……………...76.1- 86.0 points;
- Mediocre exterior…………66.1- 76.0 points;
- Poor exterior……………….50.1- 66.0 points;
- Bad exterior………………below 50.0 points;

Statistical analysis. Results were 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 mean) and the t-test (Student) to determine the significance of the difference between means. Differences were considered statistically significant at P<0.05 and indicated by specific superscripts.

RESULTS AND DISCUSSIONS

The weight of lambs at birth is considered by many specialists (Pădeanu, 2001; Călin, 2004; Pascal, 2007) to be a very important parameter in meat production, because it influences the speed of growth, the state of health, daily weight gain, total weight gain, feed utilization capacity, as well as the subsequent performances achieved by lambs in meat production.

The determination of the body weight, at birth, of the lambs from the two batches of sheep (Table 1), which constituted the experimental material, was carried out taking into account the breed of the animals, the type of birth and the sex of lambs.

Table 1. The evolution of body weight of lambs from single births depending on breed and sex in the period 0-60 days (kg/head)

No.	Age of lambs	Breed	Simple births							
			Males				Females			
			n	$\overline{X} \pm s_{\overline{X}}$	S	CV	n	$\overline{X} \pm s_{\overline{X}}$	S	CV
1.	Birth	Țurcană	15	3.999±0.137[a]	0.530	13.27	15	3.508±0.119	0.463	13.20
		Țigaie	14	4.512±0.134[a]	0.501	11.10	14	3.951±0.119	0.446	11.29
2.	30 days	Țurcană	15	10.042±0.388	1.502	14.96	15	9.274±0.356	1.377	14.85
		Țigaie	14	11.178±0.384[a]	1.439	12.87	14	10.145±0.363	1.359	13.40
3.	60 days	Țurcană	15	16.458±0.467	1.807	10.98	15	15.380±0.561	2.172	14.13
		Țigaie	14	17.584±0.558	2.087	11.87	14	16.501±0.432	1.617	9.80
4.	Total score	Țurcană	15	84.13±1.22	4.71	5.60	15	82.20±1.50	5.81	7.07
		Țigaie	14	87.50±1.71	6.60	7.55	14	86.07±1.49	5.79	6.72

[a, b]Within a row, means without a common superscript differ (P<0.05)

In the case of single births in males, the highest calving weight was achieved by the Țigaie breed, the rust variety, of approx. 4.512±0.134 kg with approx. 12.82% higher than the weight achieved at birth by the males of the Țurcană breed, between the two batches registering significant differences in this parameter;

The highest weight of females from single births was also achieved within the Țigaie breed, where an average calving weight of approx. 3.951±0.119 kg, higher weight by more than 12.00% compared to the value of the same parameter recorded in the female youth from the Țurcana breed and between these batches being significant differences.

With regard to the weight of the researched material at the age of 30 days, the best results were obtained by the male lambs from the Țigaie breed 11.178±0.384 kg, a weight higher than that achieved by the males from the Țurcană breed from births simple, and in the female superior results were achieved also by the biological material from the Țigaie breed 10.145±0.363 kg with almost 1 kg more than the weight achieved by the females from the Țurcană breed, at the same age.

Among the groups of males, the highest body weight, at the time of weaning, was achieved by the males of the Țigaie breed: 17.584±0.558 kg, which showed a superior capacity to capitalize on additional feed and recorded a higher growth rate with 6.8% compared to males from the Țurcana breed, who recorded a lower rate of growth, achieving, at the end of the experimental period (16.458±0.467 kg), a lower body weight by approx. 1.12 kg;

The batches of females from single births achieved lower weights than the males, the differences being approx. 7% in the Țurcană breed, respectively 6.5% in the Țigaie breed. Between the two batches of females there were differences of approx. 7.28% in favor of the batch of Țigaie breed males.

The best aptitudes for meat production, highlighted by the score obtained, are achieved by the lambs from the Țigaie breed. The batches of males and females from single births have a "very good exterior" exceeding 86 points, achieving a higher score by approx. 4 %, in the case of males and by approx. 5% in the case of females from the Țurcana breed, which fell into the "good exterior" classification.

The differences between males and females from single births, obtained as a result of the present research on the studied biological material (Table 2), were within the data presented in the specialized literature (Răducuță et al., 2001; Călin, 2004; Pascal, 2007).

Table 2. Significance of differences between males and females, relative to breed of lambs from single births

Specification	Țurcană	Țigaie	Difference (kg)	Calculated t value	Tabular t	Significance of differences
Males	3.999	4.512	0.513	2.67	2.46	*
Females	3.508	3.951	0.443	2.52	2.46	*

NS - non-significant differences (P>0.05); *significant differences (P<0.05); **distinctly significant differences (P<0.01); ***highly significant differences (P<0.001).

Table 3. The variation of the average daily gain of lambs from single births according to breed and sex (kg/head)

No.	Period	Breed	Single births							
			Males				Females			
			n	$\overline{X} \pm s_{\overline{X}}$	S	CV	n	$\overline{X} \pm s_{\overline{X}}$	S	CV
1	0-30 days	Țurcană	15	0.201±0.012	0.047	23.46	15	0.192±0.013	0.051	26.66
		Țigaie	14	0.222±0.014	0.055	24.96	14	0.206±0.012	0.050	24.22
2	31-60 days	Țurcană	15	0.207±0.013	0.053	25.84	15	0.197±0.023	0.091	46.38
		Țigaie	14	0.217±0.017	0.066	30.40	14	0.209±0.019	0.072	34.77

$^{a, b}$Within a row, means without a common superscript differ (P<0.05).

In the interval of 0-30 days, the lambs from the Țigaie breed from single births achieve the highest gains between 0.222±0.014 kg/head/day for males, respectively 0.206±0.012 kg/head/day for females (Table 3). The daily gain achieved by the Țurcana breed are lower, with approx. 9.4% in males and with approx. 6.8% for females.

During the period of 31-60 days, the lambs from the Tigaie breed, from single births, maintain the same higher rate of growth, achieving the highest gains between 0.217±0.017 kg/head/day for males, respectively 0.209±0.019 kg/head/ day in females.

The average daily gain achieved by the lambs from the experimental groups falls within the data contained in the specialized literature (Taftă, 2008; Voia, 2005; Pădeanu, 2011).

Table 4. The variation of body weight at calving of lambs from twin births depending on breed and sex in the period 0-60 days (kg/head)

No.	Age of lambs	Breed	Twin births							
			Males				Females			
			n	$\bar{X} \pm s_{\bar{X}}$	S	CV	n	$\bar{X} \pm s_{\bar{X}}$	S	CV
1.	Birth	Ţurcană	15	3.585±0.115	0.446	12.45	15	3.048±0.140	0.544	17.85
		Ţigaie	14	3.902±0.132	0.494	12.66	14	3.483±0.125	0.469	13.46
2.	30 days	Ţurcană	15	9.327±0.340	1.316	14.12	15	8.554±0.396	1.536	17.95
		Ţigaie	14	10.177±0.410	1.534	15.07	14	9.488±0.508	1.903	20.06
3.	60 days	Ţurcană	15	15.618±0.355[a]	1.377	8.82	15	14.493±0.389	1.508	10.41
		Ţigaie	14	16.627±0.532	1.991	11.97	14	15.831±0.531	1.986	12.54
4.	Total score	Ţurcană	15	80.50±1.42	5.48	6.81	15	77.27±1.75	6.79	8.78
		Ţigaie	14	83.21±1.50	5.80	6.97	14	80.50±2.22	8.58	10.66

[a, b]Within a row, means without a common superscript differ (P<0.05).

The males of the Ţigaie breed achieved a weight of approx. 3.902±0.132 kg, higher weight by approx. 8.84 % compared to the weight achieved at calving by males of the Ţurcană breed, originating from twin births (Table 4).

Females from the Ţurcană breed recorded a birth weight lower by approx. 12.5% compared to the weight achieved by the female youth from the Ţigaie breed, which obtained an average calving weight of approx. 3.483±0.125 kg, weight similar to that found by other authors (Răducuță et al., 2001; Călin, 2004).

Regarding the body weight, at the age of 30 days, of the lambs from the experimental batches originating from twin births, it appears that the only batch of lambs that exceeds the body weight of 10.0 kg is represented by the young male sheep from the Ţigaie breed, which achieved a weight of 10.177±0.410 kg superior to all other lots.

The highest body weight, at the age of 60 days, of the lambs from twin births was achieved by the male biological material from the Ţigaie breed, which achieved a weight of 16.627±0.532 kg, more than 1.0 kg higher the weight achieved by the male lambs from the Turcana breed. In females, the results are similar, the difference between the batches being approx. 1.34 kg, respectively 9.2% (Figure 1).

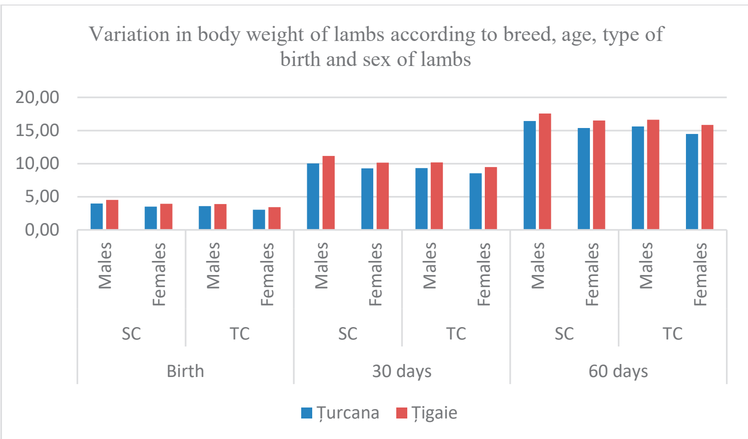

Figure 1. Body weight variation of lambs depending on different influencing factors

Table 5. The variation of body weight at calving of lambs from twin births depending on breed and sex in the period 0-60 days (kg/head)

No.	Period	Breed	Twin births							
			Males				Females			
			n	$\bar{X} \pm s_{\bar{X}}$	S	CV	n	$\bar{X} \pm s_{\bar{X}}$	S	CV
1.	0-30 days	Ţurcană	15	0.191±0.013	0.051	26.96	15	0.183±0.013	0.053	28.90
		Ţigaie	14	0.209±0.014	0.055	26.56	14	0.200±0.016	0.063	31.71
2.	31-60 days	Ţurcană	15	0.200±0.018	0.070	35.18	15	0.190±0.015	0.059	31.01
		Ţigaie	14	0.212±0.019	0.073	34.74	14	0.205±0.025	0.093	45.59

a, b Within a row, means without a common superscript differ ($P<0.05$).

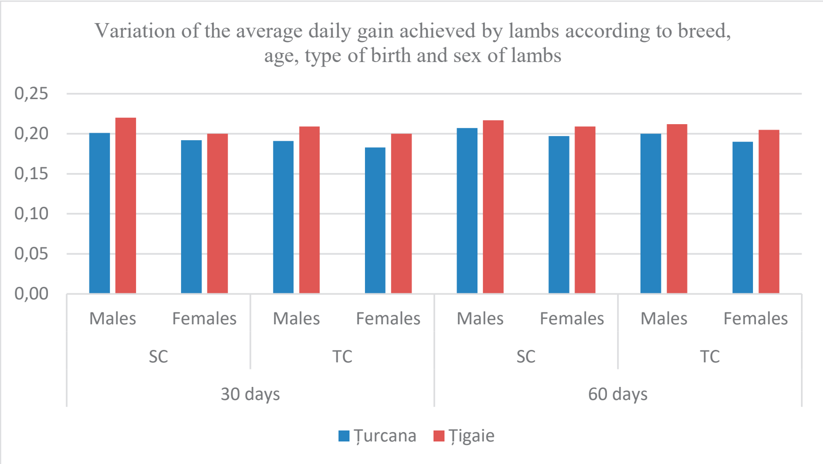

Figure 2. The variation of growth spurt in lambs depending on different influencing factors

In the case of batches originating from twin births, the lambs from the Ţigaie breed achieve a higher score, both for males and females, for this parameter, no statistically guaranteed differences were found between the scores achieved by the lambs from the two breeds.

In the interval of 0-30 days, the male youth of the Ţigaie breed, originating from twin births, achieves the greatest daily gain of approx. 0.209±0.014 kg/head/day being higher by approx. 9.4% of the increase achieved by the male lambs from the Ţurcană breed, and the females from the Ţigaie breed maintain the same trend, achieving a greater increase by approx. 9.0% compared to females from the Ţurcană breed (Table 5, Figure 2).

Within the 31-60 days growth interval, the best results were obtained by the young male and female sheep from the Ţigaie breed, the differences compared to the experimental batches belonging to the Ţurcană breed being superior by approx. 6% in the case of males, respectively with approx. 7.9% in the case of females.

CONCLUSIONS

The performances achieved by the lambs from the Ţigaie breed, in the direction of meat production are superior to those achieved by the lambs from the Ţurcană breed, both in terms of body weight achieved at different age intervals, and in terms of average daily gain and total growth rate.

Compared to the type of birth, regardless of the breed, the lambs from single births achieve superior productive performances to the groups of animals from twin births. Regarding the sex of lambs, the best results were obtained by males both in the Ţigaie and in the Ţurcană breeds.

From the phenotypic assessment of the aptitudes for meat production, it follows that the lambs from the Ţigaie breed, in good conditions of growth and exploitation, can achieve higher scores, which allow them to be placed in the class with "very good" appearance, which creates the premises for obtaining "good" and "very good" quality carcasses.

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RESEARCH ON THE METABOLIC PROFILE OF BUFFALO COWS FROM THE ROMANIAN BUFFALOES BREED, PRE AND POST-PARTUM

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Abstract

The present study aimed to monitor the changes in metabolic profile during the pre- and post-partum period of high milk producing buffalo cows and their relationship with herd management. The transition period is defined as the period from 3 weeks before calving to 3 weeks after calving. Among the factors that influence the metabolic profile of buffalo cows, the following were highlighted: nutrition, reproduction and climatic factors. This study was carried out on two batches of 10 buffaloes in different stages of the gestation period. The buffaloes benefited from the same fodder ration before going out to graze. After going out to the pasture, the animals taken in the study were fed only with grass from the natural pasture. Ante partum and post-partum blood samples were collected. The samples were analyzed biochemically. The results draw attention of the metabolic profile of the blood in evaluating the nutritional status of buffalo cows and ensuring good health in very demanding physiological conditions, in addition, they provide some indications that buffaloes are more resistant to metabolic disorders in the post-partum period.

***Key words**: buffalo cows, metabolic profile, transition period.*

INTRODUCTION

In Romania, the buffalo entered with the invasion of the Huns and Avars in the Carpatho-Danubian area. It found the good pedo-climatic conditions and so, in our country, has developed a buffalo population which had its own evolutionary path as a result of reproductive isolation (Vidu et al., 2008).

Romania has the major advantage of the existence of a research station in the field of buffalo breeding, which has an extremely valuable breeding nucleus. Increasing the economic efficiency of buffalo production and developing a breeding program are keys to actively conserving of this genetic structure. The buffalo is a species with remarkable quality, of which we can remember: high percentage of milk fat, meat with exceptional taste qualities, resistance to diseases and heavy environmental conditions, good valorization of poor quality feeds.

Immediately before parturition as well as during the first stage of lactation, increased mammary gland activity results in energy deficiency and increased lipomobilization from body reserves. Many authors investigated the buffalo metabolic response to lactation since buffaloes show a different pattern compared to other ruminants as demonstrated by the low incidence of metabolic disorders.

The centre of animal physiology is the homeostasis of the glucose, which involves primarily the somatotropine, insulin, glucagon and glucocorticoids with thyroid hormones being usually decreased after calving, with the aim to reduce tissue metabolism in order to have a higher nutrient availability for mammary gland metabolism.

The transition period is defined as the period between 3 week before to 3 week after parturition, and it is critically important to the health and profitability of dairy cows as well as in buffaloes. This phase is characterized by major physiological, nutritional, metabolic, and immunological changes. Buffaloes adjust their metabolism to deal with the considerable increase of energy and nutrient requirements needed for milk production which makes them susceptible to negative energy balance (NEB).

The metabolic adaptation to NEB requires interactions with different energy resources, and its failure may occur in various tissues such as the liver, adipose tissue, and others. Metabolic disorders in clinical or subclinical form are manifestations of the animal's inability to manage the greater metabolic demands. The metabolic response to lactation of buffaloes shows a different pattern compared with other ruminants, as demonstrated by the low incidence of metabolic disorders.

Unlike lipid metabolism, protein metabolism is not markedly influenced by the energy-protein content in diets or by different environmental conditions. When the protein level of the diets is high, animals enhance gluconeogenesis by amino acids from protein degradation; on the contrary, when the protein level of the diets is low, animals reduce production (meat and milk) and afterwards enhance hepatic protein synthesis and the production of microbial protein which may represent a significant part of total amino acid entering the small intestine of host animals. Thus, microbial protein contributes to satisfying the protein requirement of the animal for tissue maintenance and growth and for milk and wool production.

However, as reported for different buffalo species, a NEB is still one of the major concerns that may decrease the productivity in these ruminants and predispose to other pathologies and fertility disorders.

The metabolic profile test in dairy animals is used to assess nutritional status, predict the occurrence of metabolic diseases, diagnose diseases, and assess animal fertility status (Ingraham & Kappus, 1988). Among the factors that influence the metabolic profile in buffaloes, the following were highlighted: nutrition, reproduction, age, sex and climatic factors (Patel et al., 2016; Enculescu et al., 2017). A food deficiency causes metabolic, endocrine and nervous disorders, disrupting hypothalamic-pituitary-ovarian activity, with negative effects on the reproductive process, oogenesis and folliculogenesis, extending the postpartum anestrous period and decreasing fertility indices in cows (Fabry, 1993; Ferguson, 1991; Ghergariu, 1990).

Data on the metabolic profile test in Romanian Buffalo are limited, as there are no available references on normal blood metabolite values (Serdaru et al., 2011). In this metabolic profile research is necessary to continue on a larger number of animals with different sex, age, physiological state and health status (Enculescu et al., 2017).

Immediately before parturition as well as during the first stage of lactation, increased mammary gland activity results in energy deficiency and increased lipomobilization from body reserves. Despite the action of homeostatic mechanisms to maintain blood parameters within physiologic levels, changes in metabolites and hormones occur as a result of increased metabolic demands during both pregnancy and lactation (Ingraham & Kappus, 1988; Fabry, 1993). These changes are not necessarily indicative of diseases but make pregnant animals physiologically unstable and more susceptible to a number of metabolic diseases at this stage than during other life periods compromising productivity (Ferguson, 1991). Homeostasis control involves maintenance of physiological equilibrium or constancy of environmental conditions within the animal. Homeostasis is the orchestrated or coordinated control in metabolism of body tissues necessary to support a physiological state (Ghergariu, 1990). Peripartum period represents a critical life phase in buffaloes as well, since have to adjust metabolically to the increase in energy and nutrient requirements needed to ensure milk production (Ghergariu, 1990). Many authors investigated the buffalo metabolic response to lactation since buffaloes show a different pattern compared to other ruminants as demonstrated by the low incidence of metabolic disorders. The centre of animal physiology is the homeostasis of the glucose, which involves primarily the somatotropine, insulin, glucagon and glucocorticoids with thyroid hormones being usually decreased after calving, with the aim to reduce tissue metabolism in order to have a higher nutrient availability for mammary gland metabolism.

In all species glucose is used by various tissues and organs for free energy (i.e. ATP) production. In addition, glucose may be converted either into glycogen or triacylglycerols which are subsequently stored within tissues (liver, adipose tissues, muscles) or into lactose which is subsequently incorporated into milk in the case of lactating females. The destination of glucose is regulated by various hormones such as insulin, cortisol, glucagone, somatotropin and adrenalin, and consequently blood glucose levels depend on the nutritive values of the diets, on social or environmental stress conditions as well as on physiological phases.

It has been reported that nutritional deficiency is the important factor responsible for infertility in buffaloes.

As minerals and trace elements such as copper, cobalt, manganese, zinc, etc., play important role in the proper functioning of the genital organs and related activities (Fabry, 1993). Trace elements may function as cofactors, as activators of enzymes or stabilizers of secondary molecular structure (Ghergariu, 1990). Buffaloes are frequently subjected to severe dietary deficiencies of trace elements such as copper, cobalt, selenium, iodine, manganese, and zinc.

In buffalo species calcium excesses could alter the Ca/P ratio during the dry milk period, inducing parathyroid hypoactivity which would cause magnesium to increase and calcium to decrease at the beginning of the lactation due to a non immediate calcium mobilization by the bones. The altered Ca/Mg ratio favours utero-vaginal muscular release, responsible for uterus atony and eventually uterine prolapse Concomitant infertility in buffalo is believed to be associated with enzymatic dysfunctions resulting from these deficiencies (Ghergariu, 1990). Optimum protein level is necessary for the development of endocrine and sex organs.

Deficiency of trace minerals especially calcium, phosphorus and magnesium also influence the ability of animals to utilize other micro-minerals. The influence of these minerals on certain enzyme system may affect reproductive efficiency.

MATERIALS AND METHODS

The aim of this work was to investigate the metabolic profile of buffaloes with disorders of the reproductive cycle and in which estrus was induced through therapeutic protocols, compared to buffaloes that exhibited normal estrus.

The study was carried out at the Research and Development Station for Buffalo Breeding, Sercaia, during 2020, on buffaloes. The Development Research Station for Buffalo Breeding, is located on the national highway DN 1, at kilometer 223, in the territory of the Șercaia municipality, between the towns of Șercaia and Mândra, in the western part of the town of Șercaia, 56 km from the municipality of Brașov. From a mathematical point of view, it falls within 450 11'02"- 450 53'52" northern latitude and 250 06'18"- 250 11'02" eastern longitude. S.C.D.C.B. Șercaia was established in 1981 and it was populated with young female colected from all Romania, also a number of 42 bufflaoes, Murrah breed, was imported from Bulgaria (males and females) at the end of 1983 the herd reached 720 heads.

The herd of buffaloes, from its inception until now, has been the study base for research, the results of which have practical applicability in all buffalo breeding areas in the country, mainly through the diffusion of breeding material in the areas of influence.

Forty female buffaloes, aged between 7 and 13 years, in good maintenance condition and in different physiological states, were divided into two groups (20 buffaloes/group). The first group consisted of buffaloes with disorders of the reproductive cycle that are more than 60 days after calving (active anestrus caused by the presence of the corpus luteum and passive anestrus without the corpus luteum on the ovary) to which specific induction treatments were applied and estrus synchronization. The second group consisted of female buffaloes that showed normal oestrus after calving.

The buffaloes in both groups were maintained both in free housing and in the shelter being fed uniformly with green fodder, hay, corn silage and concentrates. When artificial insemination was performed, blood samples were collected. Blood samples (5 to 10 mL) were taken from the jugular vein in the morning. Blood was transported to the laboratory on ice, where serum was extracted and stored at -20ºC until further analysis. From the serum samples, the following were determined by spectrophotometric and enzymatic colorimetric methods: haemoglobin (g/dL), total serum protein (g/dL), phosphorus (mg/dL), calcium (mg/dL), alkaline phosphatase (UI/L), magnesium (mg/dL), cholesterol (mg/dL), glucose (mg/dL), lipase (UI/L) and urea (mg/dL), using commercial kits (Span Diagnostics) according to protocol the manufacturer.

Data obtained from biochemical estimations were expressed as mean ± standard error (X ± SD) and coefficients of variation (CV) for each group of buffaloes. The data were statistically analyzed by applying the Student's t-test to obtain the significance of the difference of the mean values of the two groups ($p < 0.05$ was considered statistically significant and 95% confidence).

The experimental procedures were carried out in accordance with the Romanian Legislation no. 43/2014 and Council Directive 2010/63/EU on the protection of animals used for scientific purposes.

RESULTS AND DISCUSSIONS

The results of laboratory determinations in buffaloes from the two groups are presented in Table 1.

Table 1. Results of blood biochemical parameters in buffaloes from the two experimental groups

Blood parameters	Group 1 N = 20		Group 2 N = 20		t-test
	X ± SD	CV%	X ± SD	CV%	
Hemoglobin (g/dL)	9.73± 1.02	14.37	12.68± 0,73	20.12	p < 0.05
Total serum protein (g/dL)	6.12± 0.36	22.16	8.40±0.56	17.46	p < 0.05
Calcium (mg/dL)	10.52± 0.35	24.35	8.28±0.37	18.27	p < 0.05
Phosphorus (mg/dL)	3.64± 0.48	18.42	4.27±0.77	11.31	p < 0.05
Magnesium (mg/dL)	3.06± 1.22	6.83	3.63±1.32	8.46	ns
Alkaline phosphatase (UI/L)	181.95 ± 53.17	23.17	175.19± 48.29	27.36	ns
Cholesterol (mg/dL)	98.52± 12.63	14.72	93.40± 10.84	18.57	ns
Glucose (mg/dL)	42.18± 2.62	34.58	54.18± 4.95	26.12	p < 0.05
Lypase (UI/L)	7.76± 2.45	14.38	7.12±2.90	16.74	ns
Uree (mg/dL)	40.80± 5.77	46.17	38.20± 4.49	35.92	ns

X ± SD = mean ± standard error; CV = coefficient of variation; t-test = Student's t-test; ns = not significant ($p > 0.05$).

The hemoglobin level was 9.73 ± 1.02 g/dL in group 1 and 12.68±0.73 g/dL in group 2. The results showed a significant variation ($p < 0.05$) in the hemoglobin level in buffaloes that showed normal estrus before of buffaloes with prolonged anestrus. The higher hemoglobin concentration in buffaloes that exhibited normal estrus can also be attributed to the fact that an animal requires more oxygen in any stressful condition and consequently the hemoglobin concentration can rise (Fagiolo et al., 2004). Although the level of hemoglobin does not directly influence reproductive disorders, still a low value of it could indirectly affect the functioning of the reproductive organs. A low level of hemoglobin influences the oxygenation of the tissues of the reproductive organs, which in turn could affect the cycling (Sharad et al., 2010).

Total serum protein recorded values of 6.12 ± 0.36 g/dL in group 1 and 8.40 ± 0.56 g/dL in group 2. A significant difference ($p<0.05$) was recorded in buffaloes in group 2. Protein deficiency causes uterine subinvolution which leads to prolonged anestrus after calving. Excess protein increases the incidence of retained fetal adnexa, genital infections and low fecundity. Serum calcium was 10.52 ± 0.35 mg/dL in group 1 and 8.28 ± 0.37 mg/dL in group 2 and was within the limits described in the specialized literature by other authors (8-10.8 mg/dL) (Ghergariu, 1990; Sharad et al., 2010; Enculescu et al., 2017). A significantly greater difference in calcium concentration ($p < 0.05$) was recorded in buffaloes from group 1. Serum phosphorus level in anestrous buffaloes (3.64 ± 0.48 mg/dL) was significantly lower ($p<0.05$) than that of buffaloes with normal cycle (4.27 ± 0.77 mg/dL). The lower calcium level in G2 buffaloes is influenced by the existence of lactation (Patel et al., 2016). Ca:P ratio for buffaloes with normal cycle was lower (1.94:1) compared to buffaloes with anestrus (2.89:1). Ca:P ratio should be 2:1 for better reproduction (Sharad et al., 2010). Serum magnesium (G1 - 3.06 ± 1.22 mg/dL; G2 - 3.63 ± 1.32 mg/dL) was within the physiological limits described in the specialized literature for buffaloes (1.8-3.8 mg/dL) (Ghergariu, 1990; Patel et al., 2016; Sharad et al., 2010; Enculescu et al., 2017). Magnesium plays a vital role in the metabolism of carbohydrates, lipids, nucleic acids and proteins. Alkaline phosphatase (ALP) (G1 - 181.95 ± 53.17 IU/L; G2 - 175.19 ± 48.29 IU/L), had values within the physiological limits in buffaloes (Ghergariu, 1990). The individual cholesterol values in buffaloes from both groups did not have significant differences (G1 - 98.52 ± 12.63 mg/dL; G2 - 93.40 ± 10.84 mg/dL), the reference range in the specialized literature being 73-280 mg/dL (Ghergariu, 1990; Patel et al., 2016; Enculescu et al., 2017). In the present study, buffaloes with normal cycle had significantly higher glucose concentration - $p<0.05$, (54.18 ± 4.95 mg/dL) compared to buffaloes with anestrus (42.18 ± 2.62 mg/dL). Several authors have supported the opinion that glucose concentration reflects the energy status and reproductive activity of animals (Sharad et al., 2010; Popa, 2018). Hypoglycemia influences ovarian activity in animals by reducing the release of gonadotrophins from the hypothalamus (Sharad et al., 2010). Blood glucose variations in buffaloes are related to cycling and fertility (Sharad et al., 2010). Serum lipase level was 7.76 ± 2.45 IU/L in G1 buffaloes and 7.12 ± 2.90 IU/L in G2 buffaloes. Reference values of serum lipase are not presented in the specialized literature for buffaloes. Lipase is a key enzyme in triglyceride metabolism. Urea recorded values in the physiological thresholds (21-53 mg/dL) in buffaloes from both experimental groups.

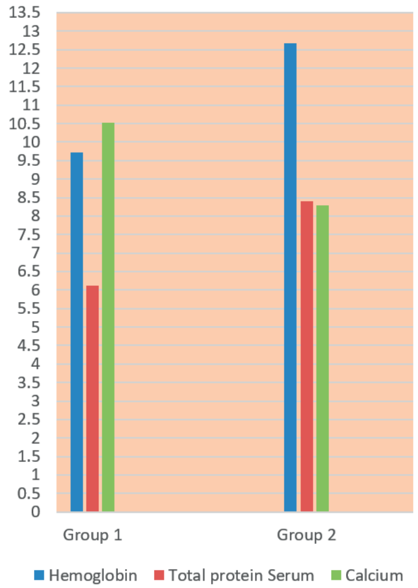

Figure 1. Results regarding hemoglobin, total protein serum and calcium

CONCLUSIONS

The metabolic profile is a beneficial means that has progressed over time. This progression or adaptation is very important to be consider for changes in feeding management.

Serum biochemical and haematological reference values are used to establish normality and to diagnose disease and physiological alterations. It could be concluded that reference values for haematological and serum biochemical constituents in lactating buffaloes were estimated in this study, these values will be helpful in interpreting laboratory results, diagnosis of diseases, and checking the health status.

From the present study it can be concluded that the levels of haemoglobin, serum proteins, glucose, calcium, inorganic phosphorus play an important role in the reproduction of buffaloes. The Ca:P ratio should also be close to 2:1 for better reproductive system activity. Subclinical nutrient deficiencies are a cause of clinical anestry in buffaloes.

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STUDY ON SEVERAL BODY DIMENSIONS OF HORSES FROM FURIOSO-NORTH STAR BREED

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Abstract

The research was conducted in 2022, on 89 horses; the studied breed was Furioso-North Star, from Rușețu Stud Farm, and 66 broodmares (from the 2001-2019 generations) and 23 stallions (from the 1999-2019 generations) were included in this analysis. Seven of these males have served as sires, while the other 16 were used for public breeding. The horses were assessed based on data obtained from standard measurements (height at the withers, heart girth, and cannon girth) performed during the annual ranking procedure. The results obtained regarding the height showed that it registered average values ranging between 161.29 ± 0.23 cm for broodmares and 161.43 ± 0.39 cm for stallions. The heart girth had average values of 183.73 ± 0.29 cm for broodmares and 185 ± 0.40 cm for stallions, while the cannon circumference had average values of 20.83 ± 0.1 cm for females and 21.52 ± 0.23 cm for males. Based on these results, it was observed that the studied group is homogeneous concerning all three analysed traits. The data obtained from the measurements are within the breed`s standard and justify the promotion/maintenance of these horses within the stud farm`s broodstock.

***Key words**: broodmares, dimensions, horses, stud farm.*

INTRODUCTION

The Furioso North Star horse breed, also known as the "half-blood" was created at the Mezőhegyes Stud in Hungary, in the early 19th century by merging two families of English half-blood horses, founded by Furioso and North-Star stallions (Klein et al., 2022).

The breed exhibits intermediate characteristics for both carriage and riding. Furioso-North Star horses are distinguished by an elegant and imposing bearing. They have a harmonious conformation and a rectangular lateral body shape. They have a relatively large development (the height ranges between 164-166 cm and a body mass of 550-600 kg) and a robust constitution, with wide, broad base and joints (Doliș et al., 2023).

In 1919, an important population of Furioso-North Star horses (44 broodmares, 7 Furioso-North Star stallions, supplemented by 2 Thoroughbred stallions) was established at the Bonțida Stud in Cluj County, Romania, from the Mezőhegyes Stud (Mureșan & Daniel, 2003).

In the time period of 2000-2004, the herd of Furioso-North Star was raised at the Jegălia Stud, and starting 2004 to 2010, it was relocated to Slatina Stud. Later, for a brief period of only one year, it was relocated at the Beclean Stud. Since 2012, the Furioso North Star population has been raised at the Rușețu Stud, in Buzău County.

Over time, specialists involved in the breeding activity have continuously sought to improve the quality of the horses, especially those comprising the reproduction herd (broodmares). Through this study, we aim to contribute as much as we can to revealing the morphological aspects influencing the performance of this breed. Considering the limited and outdated data on the evolution of the Furioso-North Star population in Romania, we consider useful to conduct this research on the current reproduction population of this breed.

MATERIALS AND METHODS

The biological material consisted in 89 horses from the Furioso-North Star breed, specifically 66 broodmares (from the 2001-2019 generations) and 23 stallions (from the 1999-2019 generations).

Out of the 23 stallions, 7 served as sires, and along with the total of 66 broodmares, formed

the reproduction herd (broodmare population) at the Rușețu Stud (this situation persisted starting2012 and it ended in 2022, according to the data recorded in the Stud's Genealogical Registry). The remaining 16 stallions were used for public breeding.
The traits that were the subject of the study were the body dimensions previously mentioned in the ranking activity: height at the withers, heart girth, and cannon girth. Measurements were conducted using standard tools, as the height measuring stick and measuring tape (Georgescu & Petrache, 1990; Marginean et al., 2005).
The data obtained from the measurements were statistically processed (Georgescu & Petrache, 1990).

RESULTS AND DISCUSSIONS

The data obtained from measurements of the height at the withers have been statistically processed and centralized in Table 1.
According to these data, it can be stated that the height at the withers measurements ranged between 155 and 171 cm, with an average value of 161.33 ± 0.20 cm. Therefore, the studied population was homogeneous, with a coefficient of variation of 2.18% (CV). Data regarding the mare population are very close, with an average height at the withers of only 0.05 cm larger than the value obtained for the total population.
For mares, the height at the withers ranged between 155 and 171 cm, with an average value of 161.33 ± 0.20 cm. When comparing these data with those provided by literature from the 1970s (157.81 ± 0.38 cm) (Velea et al., 1980), when Furioso-North Star was raised at the Bonțida Stud Farm, it can be stated that in broodmares, this trait has increased on average by 3.4 cm (+2.23%).
For the stallions included in the study, the height at the withers ranged between 155 and 167 cm, with an average value of 161.43 ± 0.39 cm. Compared to mares, the average value of this dimension was only 0.10 cm larger in stallions (+0.06%).

Table 1. The values of height at the withers (cm)

Specification	Total number of horse population	Total number of breeding herd	Broodmares		Stallions			
				*	Total	Sires	*	Public breeding stallions
N	89	73	66	52	23	7	7	16
MEAN±SEM	161.33 ±0.20	161.38 ±0.22	161.29± 0.23	157.81±0 .23	161.43± 0.39	162.29 ±0.61	159.50±1 .16	161.06±0 .49
SD	3.51	3.45	3.53	-	3.51	2.63	-	3.86
CV%	2.18	2.14	2.19	-	2.18	1.62	-	2.39
MIN.	155.00	155.00	155.00	-	155.00	160.00	-	155.00
MAX.	171.00	171.00	171.00	-	167.00	167.00	-	167.00

*Velea C. et al., 1980.

The sires had an average value of the height at the withers of 162.29 ± 0.61 cm, which is 1.23 cm larger (+0.76%) than the average value for public breeding stallions, and 1 cm larger (+0.62%) than that of broodmares. Compared to the values recorded in the 1970s at the Bonțida Stud (159.5 ± 1.16 cm), the sires' height at the withers has increased on average by 2.79 cm (+1.75%).
The heart girth (Table 2) recorded absolute values, across the entire studied population, ranging between 171 and 197 cm, with an average value of 184.06 ± 0.24 cm. It can be stated that the population is homogeneous regarding this characteristic (the coefficient of variation is 2.86%).
For the broodmare population, the chest circumference was on average only 0.14 cm smaller (-0.08%).
In females, the heart girth had an average value of 183.73 ± 0.29 cm. Compared to the data obtained at the Bonțida Stud in the 1970s (188.80 ± 0.70 cm) (Velea et al., 1980), it is observed that the chest circumference has decreased on average by 5.7 cm (-2.76%).

Table 2. The values of heart girth (cm)

Specification	Total number of horse population	Total number of breeding herd	Broodmares		Stallions			
					Total	Sires		Public breeding stallions
				*			*	
N	89	73	66	52	23	7	7	16
MEAN±SEM	184.06±0.24	183.92±0.25	183.73±0.29	188.80±0.70	185.00±0.40	185.71±0.20	190.00±1.76	184.69±0.20
SD	5.26	5.57	5.72	-	3.61	3.68	-	3.65
CV%	2.86	3.03	3.11	-	1.95	1.98	-	1.97
MIN.	171.00	171.00	171.00	-	177.00	180.00	-	177.00
MAX.	197.00	197.00	197.00	-	192.00	191.00	-	192.00

*Velea C. et al., 1980.

The heart girth for the entire population of stallions had an average value of 185±1.95 cm, which is 0.94 cm (+0.69%) more than for broodmares. In sires, this characteristic had an average value higher by 1.02 cm (+0.55%) compared to public breeding stallions and by 1.98 cm (+1.08%) compared to mares. It can also be observed that over approximately five decades, the chest circumference of sires has decreased on average by 4.29 cm (-2.31%).

The cannon girth had values that fall within the breed standard, ranging between 19.5 and 25 cm, with an average of 21.01 ± 0.1 cm (Table 3). Also, in terms of this characteristic, the coefficient of variation is low (4.10%), thus, the studied population is considered homogeneous. The average value of the characteristic for mares was 20.84 ± 0.09 cm. In broodmares, the average value of the characteristic was slightly lower by only 0.1 cm (-0.48%) compared to sires.

The public breeding stallions recorded cannon girth values between 20 and 25 cm, with an average value higher by 0.85 cm (+4.06%) compared to sires and by 0.95 cm (+4.86%) compared to mares. Compared to the data obtained at the Bonțida Stud Farm in the 1970s (Velea et al., 1980), an average increase of 0.96 cm (+4.83%) is observed in mares, and a decrease of 0.14 cm (-0.67%) in sires.

Table 3. The values of cannon girth (cm)

Specification	Total number of horse population	Total number of breeding herd	Broodmares		Stallions			
					Total	Sires		Public breeding stallions
				*			*	
N	89	73	66	52	23	7	7	16
MEAN±SEM	21.01±0.10	20.84±0.09	20.83±0.10	19.87±0.09	21.52±0.23	20.93±0.09	21.07±0.25	21.78±0.12
SD	0.86	0.65	0.64	-	1.17	0.73	-	1.25
CV%	4.10	3.10	3.08	-	5.45	3.50	-	5.74
MIN.	19.50	19.50	19.50	-	19.50	19.50	-	20.00
MAX.	25.00	22.00	22.00	-	25.00	22.00	-	25.00

*Velea C. et al., 1980.

The data obtained from body measurements were also used to calculate various body indices, as: the digital-thoracic index, the bone index, and the massiveness index (Tables 4-6).

In the studied population, the digital-thoracic index (the percentage ratio between the cannon girth and the chest circumference) had values ranging from 10.37% to 13.44%, with an average of 11.34 ± 0.08% in females and 11.63 ± 0.16% in males (Table 4). Compared to the values calculated based on the average values recorded for the cannon girth and the heart girth in the 1970s (Velea et al., 1980), a slight increase is observed in the current population, by 0.82 percentage points on average in mares and 0.18 percentage points in sires.

Table 4. The values of digital-thoracic index (%)

Specification	Total number of horse population	Total number of breeding herd	Broodmares		Stallions			
				*	Total	Sires	*	Public breeding stallions
N	89	73	66	52	23	7	7	16
MEAN±SEM	11.42± 0.07	11.34± 0.07	11.34±0.08	10.52	11.63±0.16	11.27±0.07	11.09	11.79±0.08
SD	0.49	0.43	0.43	-	0.59	0.43	-	0.59
CV%	4.28	3.77	3.79	-	5.04	3.80	-	4.97
MIN.	10.37	10.37	10.64	-	10.37	10.37	-	10.99
MAX.	13.44	12.64	12.64	-	13.44	11.67	-	13.44

*values calculated based on data from Velea C. et al., 1980.

The bone index (the percentage ratio between the cannon girth and the height at the withers) had approximately similar values, ranging across the entire studied population between 11.98% and 16.13%, with an average of 12.91 ± 0.08% in females and 13.35 ± 0.20% in males (Table 5). Compared to the values calculated for the mare population at the Bonțida Stud Farm in the 1970s (Velea et al., 1980), the index recorded a slight increase of only 0.32 percentage points in mares and 0.31 percentage points in sires.

Table 5. The values of bone index (%)

Specification	Total number of horse population	Total number of breeding herd	Broodmares		Stallions			
				*	Total	Sires	*	Public breeding stallions
N	89	73	66	52	23	7	7	16
MEAN±SEM	13.03± 0.08	12.91±0.07	12.91±0.08	12.59	13.35±0.20	12.90±0.08	13.21	13.54±0.11
SD	0.60	0.40	0.39	-	0.93	0.53	-	1.02
CV%	4.64	3.09	3.01	-	6.99	4.11	-	7.50
MIN.	11.98	12.04	12.26	-	11.98	12.04	-	11.98
MAX.	16.13	14.19	14.19	-	16.13	13.75	-	16.13

*values calculated based on data from Velea C. et al., 1980.

Regarding the massiveness index (the percentage ratio between the chest circumference and height at the withers), had an average value of 113.93 ± 0.22% in mares and 114.65 ± 0.38% in sires, with limits within the studied population ranging from 106.79% to 121.66% (Table 6).

In comparison, for this index, the value calculated for the population in the 1970s (Velea et al., 1980) was higher by 5.71 percentage points in the case of mares and by 4.66 percentage points for sires.

CONCLUSIONS

Following the study conducted on the breeding population of Furioso-North Star horses from the Rușețu Stud, the following conclusions were drawn:

- The studied population was homogeneous in terms of all analysed characteristics, with coefficients of variation having maximum values of 7.5%.
- The data obtained in this study fall within the limits specified by the literature and the conformation assessment criteria for the Furioso-North Star breed.
- The studied horses exhibited appropriate development, allowing them to be promoted and maintained in the breeding herd of the stud farm, both as broodmares and sires, as well as public breeding stallions. Based on the differences in values obtained for the studied dimensions and body indices in the current population compared to those from the 1970s, a trend of increasing height at the withers (towards hypermetric development) and a reduction in heart girth can be

observed over time. This suggests the shaping of a more refined and robust constitution, with a harmonious and supple conformation. These aspects recommend the Furioso-North Star horses as excellent for sport. Regarding this aspect it can be stated that the present analysis reveals the fact that the breeding perspective was fulfilled in Rușețu Stud to increase the height at the withers and to reduce the hearth girth for the Furioso-North Star horse breed. Furthermore, the information found in the literature are outdated.

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INFLUENCE OF FEEDING LEVEL ON THE REPRODUCTIVE CAPACITY OF HEIFERS OF THE ZNAMIANSKY TYPE OF POLISSYA BEEF BREED

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Abstract

The influence of different intensities of rearing heifers of the Znamenskaya type of Polissya beef cattle breed on their growth, development, and reproductive capacity was studied. The coefficients for determining the level of heifer feeding were developed and used: 1.75 - high; 1.57 - normal. It has been experimentally established that intensively reared heifers, compared to animals in the control group, reached the optimal live weight for mating of 384 kg much earlier. From birth to mating age, they consumed 17.2% less feed units and 15.7% less digestible protein. They had better reproductive performance, the age of fertile mating was 146 days shorter and fertility rates were 6% higher than in the control group. Intensively reared heifers were 4-5 months younger at fertile mating than animals in the control group, indicating their higher early maturity. Intensive heifer rearing shortens the period from birth to calving, reduces labour and rearing costs, and increases the reproductive capacity of animals.

Key words: *early maturity, intensive growing, Polissya beef breed, repair heifers, reproductive capacity.*

INTRODUCTION

On the world food market "grass-fed" end "marble" beef is highly valued (Kryvoruchko et al., 2023; Wu et al., 2020; Dorotiuk et al., 2003). Grazing system of livestock contributes to an optimal balance of nutrients, which gives the effect of "marbling" meat. "Grass-fed" beef contains much more Omega-3 fatty acids, beta-carotene, vitamin E and antioxidant enzymes, which have anti-carcinogenic properties in comparison with beef that keep tethered and fed concentrate (Ponnampalam et al., 2021). In Ukraine, meat cattle breeding is a specialized independent branch of animal husbandry. It is most expedient to organize it in a large natural land areas (Honcharova, 2022; Dorotiuk et al., 2003). Beef cattle are used for beef production. These animals are well adapted to different climatic conditions, undemanding to feed, resistant to diseases, with consolidated heredity, early maturing, with high paying feed products and high slaughter yield (Suprun & Dovha, 2021). The Znamyansky type of Polissya meat breed of cattle with high productivity potential was bred and officially registered in Ukraine, in 2009 (Honcharova, 2022; Pochukalin et al., 2016; Savranchuk et al., 2011). This type of cattle was created by complex reproductive crossing of the following breeds: Red Steppe, Aberdeen-Angus, Simmental and Charolais (Vdovychenko & Shpak, 2012; Podriezko, 2007). This cattle is light brown in color, has a compact type of constitution, deep and wide chest, even topline, well-developed rear third of the body, strong and not very voluminous bones (Tsukanova, 2011). In general, the Znamyansky type of Polissya meat breed animals are characterized by a calm disposition and workability, good use of pastures, rough and juicy fodder (Honcharova, 2010; Dorotiuk et al., 2003). Cows of this type, in particular, are characterized by high reproductive capacity, ease of calving and fertility (Romaniak et al., 1993). The live weight of adult cows is 500–580 kg, the intensity of growth of young animals in fattening is 1000-1250 g; males at the age of 18-24 months reach 265-290 kg, the slaughter yield is 60-64%. The milk yield of cows during 8 months of lactation is 1200 kg (Podriezko & Kernasiuk, 2011). However, the population of the Znamyansky type of Polissya meat cattle breed is very small due to the low level of reproduction of the herd and the extensive breeding of repair heifers. At the same time, the conclusions of experts regarding the expediency of using different methods of raising heifers are contradictory. Researchers point out that high level of heifer feeding has a negative effect on the formation of future milk yield of cows (Foksha et al. 2021; Liu et al., 2015; Chumachenko et al., 2014; Abeni et al., 2012).

Small livestock farms are the most optimal in terms of the ability to ensure profit (Dabija et al., 2021). Using of the beef cattle breeding system involves intensive growth of animals during the milk period with a slowdown in growth after weaning from the mother cows (Poghosyan, 2021; Saenko & Kaznacheeva, 2018; Rodríguez-Sánchez et al., 2017).

The analysis of research materials accumulated up to now shows that the above provision needs to be revised and clarified. One of the main elements of intensification in the complex of measures regarding breeding work with beef cattle, is scientifically based breeding of heifers for reproduction of the herd. In meat cattle breeding the problem of reproduction is extremely important in comparison with dairy cattle breeding. After all, the only product of a beef cow is a calf that is raised before weaning (up to 6-8 months of age) (Boyer et al., 2020; Vdovychenko & Shpak, 2012).

In order to reproduce such a herd, maximum attention should be paid to the search for optimal parameters of the breeding system of repair heifers and their mating age. As a rule, heifers of meat breeds are mated at the age of two years and older, and therefore the costs of their keeping and maintenance begin to be reimbursed only at the age of three years (at the expense of obtaining offspring) (Tucho et al., 2021; Pochukalin et al., 2019; Shevkhuzhev & Smakuev, 2015). To some extent, this reduces the number and growth of the cattle population and slows down the pace of selection. Lack of an optimal system for raising young animals is a significant problem in meat cattle breeding in Ukraine. In particular, reproductive capacity of the Znamansky type of Polissya meat cattle breed is insufficiently researched. First of all, the issue of purposeful breeding of breeding heifers after weaning needs to be investigated.

Therefore, the scientific and practical substantiation of the intensity of rearing heifers of the Znamyansky type of meat breed, the timing and age of their mating, the study of the influence of these factors on reproductive capacity is an actual issue.

MATERIALS AND METHODS

Experimental part of the research was carried out in the conditions of the family farm of Znamyansky type of Polissya meat breed cattle. At the beginning of monitoring, the number of livestock here was 25 heads. The researched farm and agricultural land are located in the Kropyvnytskyi region of Ukraine. The research was made in two stages. The first stage of the research consisted in studying the conditions of keeping, feeding and exploitation of cows and heifers. Two groups of repair heifers of the Znamyansky type of Polissya beef breed cattles, 5 heads each, were selected to carry out the second stage of research.

Metabolizable energy requirement for maintenance (M_{Em}) and metabolizable energy intake (M_{Ei}) were determined according to recommendations (Honcharova, 2010; Tsiupko & Pronyna, 1989). The amount of feed energy required for maintenance metabolism was calculated based on these indicators (M_{Ef}). Hereinafter, M_{Ef} is understood as the intensity level.

$$(ME_f) = ME_i \div ME_m$$

The heifers of the experimental groups, after weaning from the mother cows, were reared according to the intensity level: I (control) - 1.57; II - 1.75. The feeding diet of the heifers in all groups, both in terms of the set of forages and their quality, was identical and balanced in terms of all necessary nutrients. At the same time, the norms for feeding young animals were observed. The estimated average daily gain of live weight from weaning to mating in heifers of the I group was 400 g, in heifers of the II group - 700 g.

Different intensities of growing heifers from birth to breeding age were achieved by introducing different amounts of wheat grain of their own harvest into feeding rations: 1.5 kg in the ration of the control (I) group; 2.0 kg in the rations of the experimental (II) group.

The reproductive capacity of heifers was assessed by the rate of fertile insemination. Those heifers that did not come into desire after

mating during the last two months were considered to be inseminated.
Heifers were weighed monthly (before feeding) during the experiment. Based on this, the live weight of animals and the calculation of average daily, absolute gains were determined.
The digital material of the experiments was processed by the method of variational statistics (Baranovskyi et al., 2017) using the MS Excel 2003 spreadsheet processor.

RESULTS AND DISCUSSIONS

According to the data obtained from the study of the conditions of keeping, feeding and exploitation of cows and heifers in the studied farm, it was determined that the animals are raised according to the technology that provides for their free grazing and untethered keeping. In the farm, the natural mating of repair heifers and cows with breeding bulls lasts for three calendar months (from April 1 to August 1). This technology of pairing beef cows and heifers ensures their calving in the period from January to April. Accordingly, the newborn calves have time to grow up and get stronger before going out to the pasture. Calves on pasture develop well and achieve high live weight before weaning.
After birth, the calves are kept with the cow on suckers, and at the age of 8 months they are weaned. A similar technological scheme for keeping calves of meat breeds is described by researchers (Honcharova & Khokhlov, 2022; Ugnivenko, 2013; Vdovychenko & Shpak, 2012). Experts indicate that in beef cattle breeding, the main principle of raising a calf in the milk period (up to 6-8 months of age) is its stay together with the cow (Gutierrez et al., 2014; Álvarez-Rodríguez et al., 2010; Grings et al., 2007).
Based on this, during the experiment we tried to create relatively favorable feeding and housing conditions for the animals. Without this, it is impossible to identify potential opportunities for the reproductive capacity of heifers.
In the research farm, the system of keeping beef cows during the stall period is unattached (on deep litter in light-type premises, with the organization of feeding and drinking on walking and feeding grounds).
Calving of cows and heifers was organized in a room equipped with individual sections (size 5.0 x 3.0 m), a feeder and a drinker. The floor here is covered with straw (the thickness of the layer is 30-40 cm). After birth, the calf stays with the mother cow and sucks her often and little by little.
Keeping a cow with a calf in a separate section helps to strengthen the maternal instinct and sucking reflex in the calf, which is especially important during the colostral period.
Cows were kept in individual cages for 3-5 days before calving and 7-10 days after, so that the calf and cow got used to it and later easily found each other. Then the calves and cows were transferred to a group section for 5 cows. Fences were made in these sections, where the calves could pass freely. Feeders for feeding calves with concentrates and a container for water are equipped here. A place made of dry straw is equipped along the perimeter of the section, a place for keeping a cow with a calf.
In the summer and in the warm periods of the year (practically from April to December), the heifers and their mother cows graze on the pasture (Figure 1).

Figure 1. A cow of the Znamyansky type of Polissya beef breed with a calf in the pasture (original photo)

In the resting places of cows with calves on the pasture, small fenced pens with canopies for calves are arranged, where they freely passed for rest, especially in the hot season or during rain, and were fed with concentrates, green mass, hay and mineral supplements.
Calves were trained to eat fodder prepared for the stall period - 3-4 weeks before stall keeping. At the same time (from the end of October to the beginning of November), calves were separated from their mothers.
In winter, the heifers were kept untethered in an adapted room with free access to the walking area, where feeders for concentrated, rough and

juicy fodder were installed. Control over metered grain feeding was carried out by keeping heifers in spacious isolated machines with feeders. The feeding ration of heifers aged 1 to 3 months consisted of mother's milk 6.1-6.6 kg/head and concentrated fodder (accustomed to them starting with a dose of 0.2-0.4 kg/head). From the age of three months, the heifers of the two groups were grazed on pastures of equivalent grass quality and were fed with concentrates (up to 1 kg/head). Animal feed was sufficiently complete: in terms of nutrients, the amount of digestible protein, the content of minerals and vitamins in the diet. (Figures 2 and 3).

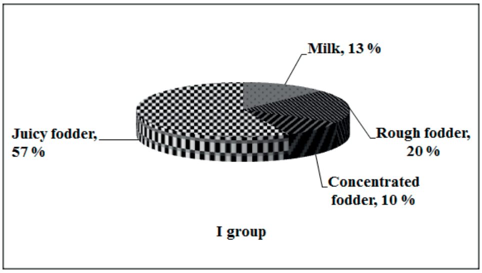

Figure 2. Fodder consumption during rearing of heifers I group from birth to breeding age per head, kg

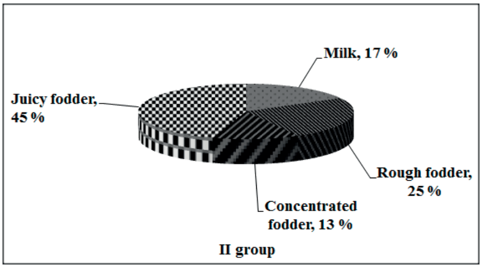

Figure 3. Fodder consumption during rearing of heifers II group from birth to breeding age per head, kg

The structure of the ration of feeding heifers of the I group from birth to mating age was as follows: milk - 13%, rough fodder - 20%, concentrated fodder - 10%, juicy fodder - 57%. In the heifers of the II group, the structure of the feeding ration, respectively, was as follows: milk - 17%, rough fodder - 25%, concentrated fodder - 13%, juicy fodder - 45%.

The total consumption of feed, taking into account the consumed milk of the mother cow, was 3232.0 feed units and 316.7 kg of digestible protein in the first control group (from birth to 20 months); and 2677.0 feed units, 267.0 kg of digestible protein in the II experimental group (from birth to 15 months), respectively.

Heifers of the II experimental group consumed less feed units and digestible protein by 555 (17.2%) and 49.7 kg (15.7%) from birth to weaning age than their peers of the I control group.

However, a higher consumption of metabolized energy was noted in the first control group of heifers from birth to mating age, - 36710.0 MJ, which is - 8093.0 MJ (22.0%) more than in the second group, and is explained by the late mating of fertile heifers in the first group.

Thus, the heifers of the second group consumed less feed daily than the animals from the first group. The total feed costs in the first group during the entire period of growing heifers until the first mating of the growing period were higher due to the extension of the growing period.

The energy requirement of animals is the amount of substances or energy that healthy animals need under optimal conditions of maintenance and balanced feeding to maintain life, obtain a set level of productivity and display reproductive function. The modern science of feeding animals provides an objective view of their need for energy and individual nutrients according to the main elements of the body's expenditure. In particular, in relation to maintaining life, development of reproductive organs during pregnancy, reproductive function in males, productivity (Freetly et al., 2023; Kharitonov & Agafonov, 2015; Gunn et al., 2015; Cardoso et al., 2014; Roberts et al., 2009).

This methodical approach to determining the energy needs of animals, combined with the results of experimental studies on the study of the characteristics of metabolism and the efficiency of the use of nutrients, makes it possible to accurately predict their productivity depending on feeding conditions and physiological state.

According to the results of our research, the highest indicators of the available metabolisable energy requirement for maintenance (M_{Em}) were established in the heifers of the II group (43.0 MJ), and the lowest in the heifers of the I group (42.4 MJ).

Metabolizable energy intake (M_{Ei}) was 66.5 MJ in the first control group, 75.7 MJ in the second experimental group. The largest amount of feed energy was observed in heifers of the II group - 1.75. In heifers of the I group, this indicator was 1.57. Animals of group I consumed less metabolisable energy requirement for maintenance per day. At the same time, the intensity of growing in animals of this group was correspondingly lower.

Thus, the analysis of spent feed testifies to the effectiveness of the proposed system of intensive rearing of heifers of the Znamyansky type of Polissya meat breed. At the same time, feed consumption is significantly reduced and the genetic capabilities of animals are used more fully.

The growth, development and reproductive capacity of animals depend, first of all, on the conditions of their feeding, maintenance and breed. The development of animals is the result of the interaction of the hereditary basis received from the parents and the specific conditions of the external environment in which it occurs. As a result, there is not only an increase in the mass of organs and tissues, but also their significant qualitative changes. In cattle breeding, great attention is paid to live weight. According to many researchers, the live weight of animals is not only a breed and constitutional feature, but also an indicator of growth and development. Of the numerous methods of directional calf rearing, the most important is the regulation of feeding level and planning of live weight gain by periods of growth and development of animals (Reis et al., 2015; Diskin & Kenny, 2014; Martin et al., 2008). Of the many methods of targeted calf rearing, the most important is the regulation of feeding levels and planning of live weight gain according to the periods of growth and development of animals (Reis et al., 2015; Diskin & Kenny, 2014; Martin et al., 2008).

The live weight of the studied heifers of the two groups at birth did not differ, but feeding during the period of suckling and their subsequent intensive rearing caused a high live weight in the animals of the II group (Figure 4).

In heifers of the II group at the age of 15 months an advantage was observed in terms of live weight by 50 kg (14.9%, P≥0.999) in comparison with animals of the control (I) group. These heifers were at a higher intensity of growing, had a live weight of 385.0 kg and were successfully mated at the age of 15 months (Figure 5). Heifers from I group reached a live weight of 384.0 kg, which is sufficient for mating only at the age of 20 months.

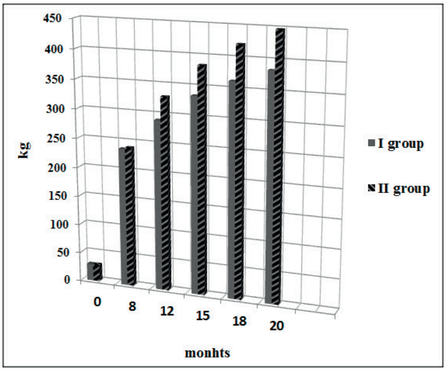

Figure 4. Live weight dynamics of heifers, kg

Figure 5. A 15-month-old experimental heifer of the Znamyansky type of the Polissia beef breed (original photo)

Thus, the relatively high live weight of intensively grown heifers of the Znamyansky type of the Polissia meat breed indicates their prematurity, a large genetic reserve, and the possibility of increasing size and stature (in terms of appropriate selection).

The heifers of the II experimental group, in terms of absolute gains, exceeded the analogue heifers of the I (control) group in all age periods. The maximum increase in absolute gains in heifers was observed up to 8 months of age. Heifers of the II group gained 356.1 kg during the period from weaning to mating (8-15 months) and exceeded the heifers of the control group by 49.9 kg (13.1%).

High average daily gains of heifers in both experimental groups were observed in the dairy growing period (from birth to weaning) and were, respectively: 853.3-865 g. During this period, the calves were kept with their mothers, they were raised according to the technology of meat cattle breeding. Weaning calves from their mothers led to a decrease in average daily gains. However, in the heifers of the II group a certain advantage was observed. Thus, they exceeded the analogs of the first control group by 300.2 g during the growing period from 8 to 12 months (68.6% P≥0.999). The same tendency took place in subsequent growing periods. It is worth noting that the growth of heifers decreased in the period of 15-18 months in individuals of both experimental groups, which coincides with the manifestation of their sexual activity (Figure 6).

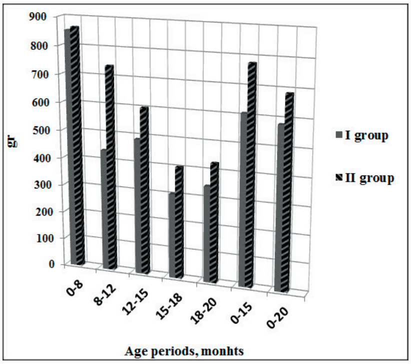

Figure 6. Average daily gains of heifers of the I and II experimental groups

During the period from birth to mating, intensively grown heifers of the II group had an average daily weight gain of 780.0 g, which is 197 g (34%, P≥0.999) more than the weight gain of the heifers of the same age from I control group.

It is known that the level of reproduction of the herd and the age of the heifers at the first insemination have a decisive influence on the economy of beef cattle breeding. In beef cattle breeding, the only product obtained from a cow is a calf for breeding or for meat. Therefore, reproductive capacity and maternal qualities of cows considered the main ones (Boyer et al., 2020; Moorey & Biase, 2020; Honcharova, 2010; Gutierrez et al., 2014; Bormann et al., 2006). In many farms heifers of meat breeds are inseminated at the age of two years and older, that is the main inhibiting factor in the development of meat cattle breeding and a certain problem in increasing the number of meat cattle. As a result, feed costs and financial costs for their growth increase. At the same time, the reproduction rate of the herd slows down significantly (Kostenko, 2012; Wathes et al., 2014; Honcharenko & Izvekov, 1998). Numerous studies: Freetly et al., 2011, Hughes, 2013, Ugnivenko, 2013, Patterson et al., 2013 have established that intensively raised heifers of meat breeds, mated at a young age (15-20 months), grow better, develop normally and fertile well. Research shows that for determining the optimal age of the first mating, it is not the age that is important, but the live weight and general development of the animals. Also, one of important factors in this aspect is the level and completeness of animal feeding and the conditions of their keeping. By balancing the use of these factors, it is possible to accelerate or restrain the growth and development of an animal at any age. Intensive feeding of heifers contributes to their earlier physiological and economic precociousness and, accordingly, faster reproduction of the herd.

We conducted a comparative study of the reproductive function of Znamyansky heifers at different intensities of growing. At fertile mating, the heifers of the II group were almost 122-152 days younger than the animals of the control group. In the heifers of the II experimental group, sexual precociousness and their ability to give the first normally developed offspring in 24-25 months are revealed. Therefore, despite the fact that heifers were mated at an early age, they had 2-3 sexual cycles. The results of the study showed that there was a difference in reproductive capacity between heifers of different groups. Live weight of heifers of all experimental groups at fertile mating was practically the same. But the heifers of the II group, with more intensive growing, reached it earlier and, accordingly, offspring were obtained from them much earlier. It was established that heifers of the II group had the largest live weight at fertile mating, which reached 385.0 ± 3.25 kg in 15 months. Heifers of the I (control) group reached the same live weight and were mated at 20 months. Heifers of intensively reared group II had a high average daily gain in the period from weaning to mating: 683.0 ± 11.20, which is 282.0 g (70.1%, P≥0.99) more than heifers of the control group. Fertilization from the first mating was higher in animals of the II group by 6 % in comparison with the control group (I). Heifers of the II experimental group had a significantly better index according to the number of inseminations. Thus, intensively grown heifers had a significant advantage according to all indicators of reproductive capacity in comparison with the group of heifers that were on standardized systems of keeping.

CONCLUSIONS

Intensive breeding of the Znamyansky type of Polissya meat breed heifers with an estimated coefficient of 1.75 provides an average daily gain of 700 g, a live weight of 385 kg at the age of 15-16 months. Under the condition of the intensity of raising heifers with an estimated coefficient of 1.57, animals have such a live weight at the age of 20 months. Intensively reared heifers of the II group were noted for their better reproductive capacity: the age of fertile mating was 146 days (P≥0.99) shorter in them, and the fertility rates were 6 % higher than those of the control group. Intensively grown heifers were younger by 4-5 months than animals of the I (control) group at fertile mating, which indicates their higher prematurity.

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THE INFLUENCE OF ALFALFA SEMI-SILAGE ADMINISTERED TO SHEEP MOTHERS IN LACTATING PERIOD ON GROWTH PERFORMANCES OF SUCKLING LAMBS

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Abstract

This study aimed to determine the influence of fodder type administered in two consecutive years (2022 and 2023) to sheep mothers in the lactating period on the growth performances of suckling lambs, to improve growth rate up to weaning. The lambs from Ţigaie breed - rusty variety (209 lambs born in 2022 and 219 lambs born in 2023) were used in the experiment from lambing up to weaning. The fodder administered to the ewes-mothers had provided a nutritional value of 198 g DP and 12.75 MJ NEM (net energy milk) in 2022, the fodder consisting of concentrates (grain corn 50%; grain barley 50%) and hill hay, while the fodder administered in 2023 is consisting of alfalfa semi-silage, hill hay and concentrates (50% grain corn; 50% grain barley) and had provided 198 g DP and 14.75 MJ NEM. The alfalfa semi-silage had significantly influenced ($p<0.001$) the growth performances of lambs in 2023 compared to those born in 2022 regarding weaning weight (19.98 kg vs. 16.82 kg), total weight gain (15.71 kg vs 12.84 kg) and average daily gain (224.44 g vs. 183.49 g).

Key words: *alfalfa semi-silage, fodder, lamb, growth rate, Ţigaie.*

INTRODUCTION

The diet administered to the animals influences the final product (Milevski et al., 2014).
The influence of alfalfa hay on the productive performance of sheep and lambs is well known. In recent years, the interest in the production of alfalfa bales for the purpose of obtaining semi-hay, semi-silage, or silage has increased a lot. By ensiling the grass, on the one hand, ensures the maintenance of nutrients and the improvement of palatability (Wang et al., 2021), and it facilitates their fair storage, there where are not enough spaces for the storage of fibrous fodder, on the other hand.
More than that, in periods and areas with heavy rains, which make it impossible to produce hay, ensiling the grass immediately after mowing or after a wilting period, remains the most accessible alternative and, at the same time, helps to obtain quality fodder necessary for ruminants for the winter period. Transforming of the surplus of feed in unfavorable periods of the year in silage can increase farm profitability (Stanley, 2003).
Fraser et al. (2002) consider that legume forage assures better animal production than silages from other grass or crops.
Yang et al. (2022) have shown that ensiling alfalfa poses challenges because of its elevated buffering capacity and limited concentrations of fermentable carbohydrates. Similar results were observed by other authors (Plaizier, 2004; Wang, 2021) who considered that making alfalfa silage without using additives or mixing with other forages can be a challenge due to its low content of water-soluble carbohydrates (WSC) and high buffering capacity. The progress over the years has led to improvement of ensiling technology, many studies (Seale et al., 1986; Pahlow et al., 2002) showed a great potential to conserve legumes forage.
Chen et al. (2013) appreciate that, by ensiling alfalfa, costs for food production are reduced, while at the same time, its preservation is simplified.
Several authors (Weinberg et al., 1993) believe that leguminous silages with a lower content of dry matter and WSC are more resistant to aerobic deterioration than cereal silages.

In the specialized literature, there is a lack of information about the influence of alfalfa simi-silage on the milk production of sheep and growth performance of lambs.
The influence of the silage in two consecutive years (2007 and 2008) on ewe performance was shown by Bernes and Stengärde (2012) and on lambs performance by Bernes et al. (2012), with the mention that, the silage was from grass-dominated crops (mainly *Phleum pratense* L.).
The objective of the study is to compare the influence of the diet from two consecutive years (2022 - diet without alfalfa semi-silage and 2023 - diet including alfalfa semi-silage) on the milk production of sheep during the lactation period and on the growth performance of the lambs from birth up to weaning.

MATERIALS AND METHODS

The research works were conducted by Experimental Laboratorium Reghin of Research Institute for Sheep and Goat Palas Constanta, Mures County, 46°46' N/ 22°42'E; 395 m altitude; annual rainfall varies between 650- 700 mm; average temperatures 19/–3°C during summer/winter).
The alfalfa semi-silage was produced at these coordinates, from the fourth year and fourth cut (the middle of September). The plants were harvested after a wilted period of 48 h and the bales were formed. After two hours, the bales were wrapped with a stationary bales wrapping machine. No additives were used for the production of alfalfa semi-silage.

Animal management

The biological material on which the experiments were carried out was constituted of adult sheep and their sucking lambs from Ţigaie - rusty variety (195 sheep and 209 lambs in 2022; 201 sheep and 219 lambs in 2023). Lambs, born between January and March, underwent initial identification and weighing (±0.1 kg) within the first 24 hours, involving the application of ear tags. Data on sex, date of birth, type of birth, and the respective dam and ram groups were documented. Subsequent weighings (±0.5 kg) were conducted at the weaning phase.
Ewes and their lambs were maintained together in identical management conditions for a duration of two months post-lambing. Weaning occurred at around 73 days in 2022 and 61 days in 2023. During the suckling phase, the lamb diet was tailored to achieve a growth potential of 300 g/head/day, adhering to NRC (2007) guidelines (135 g DP and 10.89 MJ NE). Additionally, the diet for ewes was formulated to meet the nutritional demands of late pregnancy and lactation. During the experiment were provided blocks of mineral and vitamin supplements.
The structure of concentrated fodder administered to lambs up to weaning was: 30% corn flour, 30% barley flour, 25% corn grain, 11.25%, sunflower groats, 2.25% calcium, and 1.5% salt.
The sheep mothers were kept on pastures throughout the year, and in shelter in late gestation up to lambs weaning. In this period, the feeding of ewes was constituted of concentrated and hill hay in 2022, and of concentrated, hill hay and alfalfa semi-silage in 2023 (introduced in the diet three weeks before starting to lamb).
The structure of fodder is presented in Table 1.

Table 1. The structure of fodder used to feed ewes in the lactating period

Characteristics	2022	2023
Hill hay (%)	59.96	33.31
Alfalfa semi-silage (%)	-	33.31
Corn grain (%)	19.98	16.66
Barley grain (%)	19.98	16.66
Calcium (%)	0.04	0.03
Salt (%)	0.04	0.03
Dry matter intake/day (kg)	2.60	2.09
Digestible protein g/day	198	198
NEM MJ/day	12.75	14.75

The computed composition was determined using tabular values derived from the ingredient composition of the experimental diet, as outlined in the NRC (2007) guidelines.

The lambs were measured for birth weight (BW) and weaning weight (WW).
Birth weight (BW), weaning weight (WW), weaning age, initial total gain from birth up to weaning, average daily gain (ADG), and milk production of sheep in the lactating period were determined.
After determining the initial total gain at weaning, this was recalculated for 70 days.
The milk production of ewes was determined by the Nica method (this method takes into account the fact that 1 kg of gain, achieved during the lactation period, is obtained with 4.5 kg of milk).

The statistical data processing utilized the ANOVA program, and the analysis incorporated the "Tukey" tests.

RESULTS AND DISCUSSIONS

The milk production of sheep in the lactating period (Table 2) was highly ($p<0.001$) influenced by the diet administered in 2023 compared to 2022. This characteristic is spread over the body weight evolution of lambs, total gain, and average daily gain (ADG) from birth up to weaning.

The weights of lambs at birth and weaning were significantly higher ($p<0.001$) for lambs born in 2023, compared to the ones born in 2022. No significant differences ($p>0.05$) were found between the years concerning initial total gain birth-weaning, but after its recalculation for a period of 70 days, it resulted in a significant difference ($p<0.001$) of recalculated total gain and daily gains in 2023 compared to 2022. It is to be noted that the milk production of sheep was significantly influenced ($p<0.001$) by the alfalfa semi-silage.

Table 2. Mean (± SE) for body weight evolution, total gain, and ADG of lambs from birth up to weaning

Specification	2022 (n = 209)	2023 (n = 219)	Mean differences	P_{Tukey}
Birth weight (kg)	3.98 ± 0.04^{A}	4.27 ± 0.04^{B}	-0.292	< .001
Weaning weight (kg)	16.63 ± 0.22^{A}	17.85 ± 0.21^{B}	-1.229	< .001
Weaning age (days)	73.18 ± 1.09^{A}	61.11 ± 1.07^{B}	12.072	< .001
Initial total gain birth-weaning (kg)	12.65 ± 0.20	13.59 ± 0.19	-0.937	0.007
ADG birth-weaning (g)	183.49 ± 3.67^{A}	224.44 ± 3.59^{B}	-40.948	< .001
Recalculated weight at 70 days (kg)	16.82 ± 0.27^{A}	19.98 ± 0.27^{B}	-3.159	< .001
Recalculated gain (kg)	12.84 ± 0.26^{A}	15.71 ± 0.25^{B}	-2.867	< .001
Production of suckled milk birth-weaning (kg)	57.80 ± 1.16^{A}	70.70 ± 1.13^{B}	-12.899	< .001

Means with different superscripts (A, B) in each trait differ ($p< 0.001$).

The analysis of differences recorded for the same sex in different years, highlights the fact (Table 3) that there were recorded significant differences ($p<0.001$) for all traits (except initial gain birth-weaning) in favour of the year 2023 compared to 2022.

The analysis of differences recorded between the sexes in the same years showed a greater value for male lambs both in 2022 and 2023, but only in 2022 differences were significant ($p< 0.001$).

For lambs born in 2023, significant differences were recorded significant differences ($p< 0.001$) in favor of male lambs regarding ADG birth-weaning, recalculated weight at 70 days, recalculated gain, and production of suckled milk in the birth-weaning period.

In the results disseminated from a project founded by the EU (1997-2001), Low-input animal production based on forage legumes for silage (LEGSIL), none of the three working groups (Nordic-, Maritime- and Continental Region) have included sheep as being suitable to be fed with legumes silage.

However, in recent years more researchers have studied the effect of different diets including legume silage on the performances of the and lambs.

In an experiment on weaned lambs, Sobiech et al. (2015) showed that lambs fed legume silage (red clover, alfalfa) obtain better growth performance than lambs fed grass silage.

Other experiments have studied comparatively diet based silage (grass-dominated) versus concentrate.

Thus, Bernes and Stengärde (2012) observed that it is difficult to maintain a good nutritional status in ewes with more than one lamb if they are fed only forage, without concentrates. Bernes et al. (2012) showed in an experiment on weaned lambs in two consecutive years, that weight gain was highest in both years in the lamb groups fed concentrates after weaning and lowest in the group fed only silage. The authors considered that a diet with high-nutrient-quality silage and without concentrates is difficult to meet the lamb's nutritional requirements for optimal growth.

Table 3. Mean (± SE) for body weight evolution, total gain, and ADG of lambs from birth up to weaning (depending of year and sex)

Specification	Year	Sex	n	$X \pm s_x$
Birth weight (kg)	2022	F	94	3.83 ± 0.05^{ac}
	2023		123	4.16 ± 0.06^{A}
	2022	M	115	4.10 ± 0.05^{bC}
	2023		96	4.41 ± 0.07^{B}
Weaning weight (kg)	2022	F	94	16.19 ± 0.28^{a}
	2023		123	17.42 ± 0.21^{A}
	2022	M	115	16.98 ± 0.26^{b}
	2023		96	18.42 ± 0.31^{B}
Weaning age (days)	2022	F	94	77.13 ± 4.17^{a}
	2023		123	61.73 ± 0.66^{A}
	2022	M	115	69.95 ± 1.08^{b}
	2023		96	60.30 ± 0.78^{B}
Initial total gain birth-weaning (kg)	2022	F	94	12.36 ± 0.27
	2023		123	13.26 ± 0.19
	2022	M	115	12.88 ± 0.24
	2023		96	14.01 ± 0.28
ADG birth-weaning (g)	2022	F	94	175.78 ± 5.35^{a}
	2023		123	216.32 ± 3.08^{Ab}
	2022	M	115	189.79 ± 4.46^{b}
	2023		96	234.84 ± 4.86^{B}
Recalculated weight at 70 days (kg)	2022	F	94	16.13 ± 0.40^{a}
	2023		123	19.30 ± 0.24^{Ab}
	2022	M	115	17.38 ± 0.34^{b}
	2023		96	20.85 ± 0.38^{B}
Recalculated gain (kg)	2022	F	94	12.30 ± 0.37^{a}
	2023		123	15.14 ± 0.22^{Ab}
	2022	M	115	13.29 ± 0.31^{b}
	2023		96	16.44 ± 0.34^{B}
Production of suckled milk birth-weaning (kg)	2022	F	94	55.37 ± 1.68^{a}
	2023		123	68.14 ± 0.97^{Ab}
	2022	M	115	59.78 ± 1.40^{b}
	2023		96	73.98 ± 1.53^{B}

Means with different superscripts ($^{A, B, C, a, b, c}$) in each trait differ ($p < 0.001$).

CONCLUSIONS

This study highlights the fact that the introduction of alfalfa semi-silage in the sheep's diet positively influences the milk production of the sheep during the lactation period and, implicitly, the growth rate of the lambs until weaning.

The weaning age of the lambs can be reduced, which contributes to the increase in the economic efficiency of the farms by reducing some costs during the lactation period, thus:

reducing the amount of feed required and reducing the labour for feeding animals (faster sales of the lambs and taking the ewes out to pasture).

Further, research is needed to highlight the best combinations of diets that can contribute to improving the performance of sheep and lambs during the lactation period.

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INFLUENCE OF COMPLEX MICROBIAL PREPARATION ON PRODUCTIVITY AND CLINICAL HAEMATOLOGICAL STATUS OF RABBITS KITS

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Abstract

The research aimed to study the influence of a bioactive compex microbial product, obtained from yeast biomass from beer and wine industry wastes and residual cyanobacterial biomass (spirulina) after production of medicinal remedies, on the productive parameters, health and clinical haematological status of recently weaned rabbit kits. The experiment was carried out on 3 batches of 14 kits from weaning to about 80 days of age. The rabbits in two experimental groups were supplemented with proposed preparation at doses of 3.0 and 9.0 g per 1 kg of concentrated feed per day. The use of the complex microbial preparation in the daily ration has a beneficial effect on the rabbit kits, evidenced by the general health status of the animals, reduction in the number of leukocytes and lymphocytes in the blood and the increase in the number of segmented neutrophils.

Key words*: clinical haematological status, haematopoiesis, microbial preparation, productive parameters, rabbits.*

INTRODUCTION

In recent years, rabbit farming (cuniculture) has emerged as a relatively new sub-branch of the livestock sector, which contributes essentially to the implementation of one of the most difficult tasks of the national economy - ensuring the population with products of animal origin. In addition, rabbit meat is a food product with extraordinary nutritional qualities, that is in high demand by the people, as well as is recommended to the sick persons. (Macari, 2019; Macari et al., 2017). The phenomenon of vertiginous development of rabbit farming, including Republic of Moldova, might be explained by the physiological and metabolic properties of rabbit regarding growth rate, reproduction, especially high prolificacy, accessibility of maintenance and exploitation.

However, it is widely accepted that the animal husbandry, particularly of rabbits - scarry animals, under intensive conditions can be influenced by the impact of stressors, considered unavoidable on modern livestock farms, which can negatively impact the growth process. In this context, the researchers in the fields of biology, biotechnology, veterinary medicine, animal husbandry are studying possibilities to develop and test biologically active products that are safe to animals, humans and the environment (Galip & Seyidoolu, 2012; Rotaru, 2016).

The essence of the present research was to find more knowledge and to evaluate the impact of a biologically active microbial preparation complex (CMP-4) on some parameters of clinical and haematological status, health and productivity in recently weaned young rabbits.

MATERIALS AND METHODS

The applied research, focused on testing (CMP-4), was carried out at the rabbit farm "Mațencu Dmitrii" from the village of Braviceni, district Orhei, Republic of Moldova, on recently weaned young rabbits. The study was conducted on 3 batches of rabbit kits. The following Table 1 demonstrates the principle of the experiment.

Table 1. The scheme of CMP-4 administration to rabbit kits

Batch	Number of rabbit kits	Route of administration	Dose of administration, g/kg feed supplement	Mode of administration
CB	14	-	-	
EB 1	14	peroral	3.0	daily
EB 2	14		9.0	

The object of the research was a natural complex microbial preparation (CMP-4) that contains biologically active compounds obtained from *Saccharomyces* yeast biomass from wine and beer production and *Spirulina (Arthrospira*) *platensis* biomass.

CMP-4 presents a combination of 6 biologically active extracts, 2 [β-glucan (LB-GL)] and aminoacid protein (LB-AAP) from yeast of beer production (Beşliu et al., 2022; Chiselita et al., 2022), 2 [β-glucan (SRM-GL)] and aminoacid protein (SRM-AAP) from yeast of wine production (Chiselita et al., 2023) and 2 [peptidoglycan (PPGE) and polysaccharide (PS)] from cyanobacterial biomass.

CMP-4 is obtained by the mixing of PPGE with LB-GL and SRM-GL, the gradually introducing of LB-AAP, SRM-AAP and PS in this mixture and drying at +45-50˚C for 24-48 hours. CMP-4 is a solid, dark green, spirulina-smelling liquid product with 97.08 ± 0.23% DW. and moisture of 2.92 ± 0.23%, which containins not less than 550 mg/g PPEG, 100 mg/g LB-GL, 100 mg/g SRM-GL, 100 mg/g LB-AAP, 30 mg/g SRM-AAP and 25 mg/g PS.

Yeast extracts were obtained from yeast (*Saccharomyces*) from the wastes of the production of beer and red Merlot wine. PPGE was obtained in the same technological flow from dry cyanobacterial biomass, residual after obtaining of some peptide extracts and represents the insoluble sediment, remaining after the extraction of pigments, lipids and proteoglycans, which was dried in an oven at a temperature of 50 ± 5°C to a constant mass with 7-8% moisture and grounded to a powder.

Due to its complex composition, CMP-4 contains a broad spectrum of biologically active substances - proteins, including essential and immunoreactive amino acids, β-glucans, sulphated polysaccharides, pigments, especially anthocyanins and β-carotene, macro-, microelements. It possesses a total antioxidant activity of 39.8 ± 0.15% inhibition (ABTS) and antioxidant enzymes catalase (CAT) of 813.85 ± 42.66 mmol/min/mg protein and superoxide dismutase (SOD) of 92.71 ± 2.86 U/mg protein.

CMP-4 was introduced into the basic feed using the granulator, intended for the production of dense granules. Thus, 3 type of granulated feeds were produced for rabbit kits. The feed intended for the control batch (Control batch CB) contained only basic feed, and the feed intended for the experimental batch 1 (EB 1) and the experimental batch 2 (EB 2) additionally contained CMP-4 at a concentration of 3.0 and 9.0 g per 1 kg (0.3 and 0.9%/V), respectively. The feeds were administrated to the respective batch from weaning until slaughter (Table 1).

During the experiment the young rabbit kits were continuously monitored and, periodically, body mass was measured by individual weighing. Body temperature, heart and respiratory rate per minute were determined for 5 rabbits from each batch at the start of the study, and, subsequently, throughout the period of investigation.

For laboratory tests, blood samples were taken from rabbits in two stages: at the beginning of the study, until the administration of the tested remedy, from five rabbits randomly selected, and at the end of the study, at about 80 days of age, already from 5 animals from each group. It is also important to mention that during the handling of the rabbit kits, attention was also paid to their behaviour and possible adverse reactions following the use of CMP-4.

Animals used in study of the same breed, age, body mass were housed in the same shelter, at the similar hygienic parameters. The haematological constants, determined in the blood of rabbit kits, were: Haemoglobin concentration- HgB, g/l; Total erythrocyte count- RBC, 1012/l; Haematocrit content - HCT, % - indicator representing erythrocyte volume fraction; MCV, fl - mean corpuscular volume; MCH, pg - mean erythrocyte haemoglobin; MCHC, g/l - mean erythrocyte haemoglobin concentration; total leucocyte count - WBC, 109/l. Blood samples were read on a haematological analyser: Myndrey 500. Determined marker parameters of leukocyte formula were analysed by microscopic examination with immersion objective of blood smear stained by the usual method - Romanovsch.

Statistical evaluation of clinical and haematological indices was performed using the parametric t-Student criterion with $P<0.05$.

RESULTS AND DISCUSSIONS

The obtained results demonstrated that the administration of CMP-4 to rabbit kits for a period of about 40 days did not cause any changes or deviations from the normal physiological state of the animals, which was confirmed by the physiological and metabolic tests, bio productive and haematological indices of the rabbits. The body temperature is the basic parameter of the animals' health status that is easy to monitor. The value of this clinical parameter at the beginning of the experiment was 39.51 ± 0.10°C in rabbits from the control batch (CB), +39.50 ± 0.06°C in rabbits from the experimental batch1 (EB 1) and 39.53 ± 0.06°C in rabbits from the experimental batch2 (EB 2). The values of baseline respiratory rate of rabbits of all three groups at the beginning of the study were within the range of 87.39-87.51 breaths/min and averaged heart rate - 157.08-158.54 beats/min. These results, indicate on the high animal health status in all batches on the onset of research.

The changes of haematological indices at intact rabbit kits from CB, EB 1 and EB 2 is demonstrated in Table 2.

At the end of the study red blood cell count (RBC) demonstrated a trend to increase in EB 2 by about 3% compared to the control and by 10.7% compared to the values recorded in EB 1. Haemoglobin and hematocrit levels showed lower values in EB 1 experimental batch (Table 2).

The mean corpuscular volume (MCV) demonstrated an increasing trend at rabbit kits from the control batch, constituting an increase of 3.1% compared to baseline values. Towards the end of the experiment, the tested product manifested a beneficial effect, characterized by a decrease of the MCV value by 2.0-4.8%, compared to the control (Table 2).

Table 2. Indices of haematological parameters in rabbit kits under the influence of CMP-4 (M ± m) at the start and the end of experiment

Parameter (the end of experiment)	Baseline values	Batch of animals		
		CB	EB 1	EB 2
RBC, 10^{12}/l	5.35±0.26	5.43±0.20	5.05±0.45	5.59±0.23
HgB, g/l	112.00±2.56	116.80±1.52	108.60±6.99	115.40±2.26
HCT,%	34.76±0.93	36.26±0.36	33.00±2.13	35.64±1.12
MCV, fl	65.12 ±1.69	67.14±2.42	65.78±1.74	63.90±1.47
MCH, pg	21.04±0.62	22.06±0.72	21.60±0.59	20.66±0.45
MCHC, g/l	323.00±1.70	323.00±2.32	328.80±0.89	327.00±7.15

Similar results were also recorded at rabbit kits, treated with the injectable remedy BioR (Macari, et al., 2017), as well as in rabbit kits, whose feed was supplemented with the product - ZooBioR (Macari, 2019). The possibility of decreasing of the MCV value in rabbit blood by the utilization of different biologically active products has been reported by some researchers (Ewuola et al., 2012; Ojokuku et al. 2011). Similar changes, of the investigated parameter were found in rabbits raised in a non-polluted area, compared to those raised in intensely polluted area (Kashapova, 2007).

It was established that mean corpuscular haemoglobin (MCH) values in the experimental groups were with 2.1-6.3% lower than the control, while the mean corpuscular haemoglobin concentration (MCHC) parameter values were with 1.3-1.8% higher than the control values, an undeniably beneficial phenomenon, revealing enhanced haematopoiesis in rabbit kits from both experimental groups (Table 2). A high level of MCHC was also found in rabbits receiving other bioactive remedies (Ojokuku et al., 2011; Galip & Seyidoolu, 2012; Macari, 2019). Thus, our results indicated that CMP-4 has positive impact on hematopoietic function and nonspecific resistance in rabbit kits.

The role of evaluation of the action of test product at the cellular level through the determination of the absolute number of leukocytes and the highlighting of certain aspects in the leukocyte formula cannot be underestimated. The dynamics of leukocytes and the basic components of the leukocyte formula in rabbit kits under the influence of CMP-4 are presented in Table 3.

Leukocyte count (WBC) towards the end of the experiment has demonstrated an increasing trend in rabbit kits from all batches compared to baseline values. However, at the end of the study in rabbit kits from CB the WBC value in the blood was 12.94 ± 2.19 x 10^9/l, when in EB 1 and EB 2 this value has decreased to 10.21-11.27 x 10^9/l, which represent a reduction with 12.9-21.1% (Table 3). The decrease in the total number of white blood cells in both experimental groups can be considered beneficial, as it testifies to reduction of the negative impact of stress, especially technological stress, persistent in modern rabbit breeding and farming and, also, to the anti-stress and adaptogenic properties of the tested product. Similar blood leukocyte dynamics have been reported by other authors which have tested various bioactive remedies on animals (Pistol et al., 2021; Curca et al., 2014).

Rabbit lymphocytes from CB (control) and EB 1 group exhibited an increasing trend compared to baseline values, with a maximum lymphocyte count of 63.40 ± 8.00% in CB rabbits, which was 15.7% more than baseline. At the same time, the values of this haematological indicator of rabbits from EB 1 and EB 2, which received different doses of CMP-4, are lower compared to the values recorded in CB group, the decrease being 5.0-14.5% (Table 3). Similar results of blood lymphocyte reduction in rabbit kits blood treated with another bioactive remedy obtained from *Spirulina platensis* were reported (Macari, 2019). Thus, the decrease in blood lymphocytes in EB 1 and EB 2 groups and the dynamics of other haematological indices, highlight the increase of non-specific resistance in rabbit kits blood under the impact of CMP-4 product.

Table 3. Dynamics of leukocytes and basic components of the leukocyte formula in rabbit kits at the administration of CMP-4 (M ± m)

Parameter(the end of the experiment)	Baseline values	Batch of animals		
		CB	EB 1	EB 2
WBC, 10^9/l	8.89±0.89	12.94±2.19	11.27±2.00	10.21±0.79
Limfocyte, %	54.80±0.57	63.40±8.00	60.20±10.92	54.20±0.84
Nonsegmented neutrophils,%	3.60±0.91	3.20±0.67	2.60±0.70	3.80±0.89
Segmented neutrophils,%	33.00±4.87	29.00±2.83	33.20±3.40	36.60±4.34
Eosinophil, %	0.60±0.27	1.00±0.00	0.80±0.22	0.80±0.22
Monocyte,%	8.00±1.77	3.40±1.41	3.20±1.14	4.60±1.74

Unsegmented neutrophils did not manifest essential changes, remaining at a practically constant level throughout the study in animals from all study groups (Table 3).

Segmented neutrophil values in CB group of rabbits towards the end of the experiment has demonstrated a weak decreasing trend compared to baseline values. At the same time, the values of these indices in EB 1 and EB 2 groups were higher by up to 14.5 and 26.2% respectively compared to the values determined in CB group (Table 3). Similar effect was established at the administration of medicinal preparations obtained from spirulina, a study in which the haematological analysed index was with 6.9-27.1% higher compared to the values of the control group (Macari V., 2019). Supplementation of the feed ration of EB 1 and EB 2 rabbits with CMP-4 did not induce at the end of the study a significant difference in the number of eosinophils in the blood of rabbit kits compared to the CB control (Table 3). This effect can be considered beneficial as it highlights the harmlessness of CMP-4 to the rabbit kits.

Blood monocyte levels decreased with age of rabbits from all groups trained in the experiment compared to baseline values. Thus, in CB group the number of monocytes decreased by 2.4, in EB 1 by 2.5 and in EB 2 by 1.7 times compared to baseline values. At the same time, the number of monocytes in EB 2 rabbits averaged 4.60±1.74%, which was with 35.3% more than CB and 43.8% more than EB 1 groups. Based on the obtained results, it can be mention that CMP-4, administered daily with the feed during the period of intensive rearing and fattening, was well tolerated by rabbits, demonstrated beneficial effect on haematopoiesis and non-specific resistance of the animals.

The investigation of zootechnical parameters has an important role in clarifying of the effect of CMP-4 at the rabbit organism. The dynamics of body weight indices in rabbit kits under the influence of CMP-4 are presented in Table 4.

The results demonstrated that CMP-4 had a beneficial effect on the growth and development of rabbit kits in EB 1 and EB 2. Thus, at the age of 50 days, the body weight of CB rabbits was in average 1243.94 ± 11.13 g with an addition of 384.7 g during 10 days of experiment. During the same period the weight gain of EB 1 and EB 2 rabbits was 435.0 and 420.0 g respectively and body weight was 1294.18 ± 6.36 and 1279.96 ± 16.48 g respectively, which was with 13.1 ($p<0.001$) and 9.2% more than CB values (Table 4). It must be mentioned that in the condition of stress for the animals, 10 days after weaning, the body mass of EB 1 rabbits fed with the minimum dose of CMP-4 - 3 g/kg feed, was with 3.6% higher compared to the mass of EB 2 rabbits fed with CMP-4 - 9 g/kg feed (Table 4).

At 70 days of life the growth rate in rabbits remained practically similar to previous periods. Finally, at 83 days of age, the body weight of CB rabbits was 2834.75 ± 28.26 g and that of EB 1 and EB 2 was significantly higher ($p<0.001$) by 114.4-154.1 g or 4.0-5.4% compared to the baseline (Table 4).

The indicator - total gain/period/rabbit, shows an increasing trend in EB 1 and EB 2 group. Thus, if in CB rabbit group the total gain during the experimental period was 1975.55 g, then in EB 1 and EB 2 group, whose feed was supplemented with CMP-4, it was 114.4-153.4 g or 5.8-7.8% higher than the reference value and constitutes 2089.93 and 2128.92 g, respect-tively. It can be mentioned that the total gain of rabbits in EB 2 was also with 1.9% higher than the values recorded in EB 1 (Table 4).

Table 4. Dynamics of body weight indices in rabbit kits under the influence of CMP-4 (M ± m)

Parameter	Batch of animals		
	CB	EB 1	EB 2
Number of animals/baseline, heads	14	14	14
Body weight - baseline experiment (40 days), g	859.20±4.66	859.22±3.93	859.96±3.23
Body weight - 50 days, g	1243.94±11.13	1294.18±6.36***	1279.96±16.48
Body weight - 60 days, g	1553.82±14.39	1675.21±15.51***	1687.66±9.77***
Body weight – 70 days, g	1937.75±7.62	2067.49±14.78***	2080.60±8.47***
Body weight - 83 days, g	2834.75±28.26	2949.15±10.84***	2988.88±18.84***
Total gain/period/rabbit - 43 days, g	1975.55	2089.93	2128.92
The average daily/period gain (43 days), g	45.94	48.60	49.51
Viability, %	100	100.0	100.0

Note: ** - $P<0.01$; *** - $P<0.001$

The average daily/period gain in CB rabbits was 45.94 g, and in EB 1 and EB 2 group was 48.60 and 49.51 g respectively, which was with 5.8% and 7.8% more. Amany et al. (2017) in a study conducted on rabbits found that the highest non-significant weight gain was associated with feed supplementation with dried taro (*Colocasia esculanta*) waste at (0.0, 7.5, 15 and 22.5%) and in combination with dried yeast (*Saccharomyces cerevisiae*, 0.5%) at the age of 6-14 weeks. Supplementation of rabbit feed with biologically active remedies, was studied by Macari et al. (2019). The researchers investigated the effect of ZooBioR, a spirulina product, on productivity of rabbit kits. The rations were supplemented with 2.5, 5.0 and 10.0 mg/kg feed. There was a significant effect on animal performance. The weight of a rabbit at 80 days of age in the experimental groups was 114.3-264.3 g, or with 3.9-8.5% higher than the control group ($P<0.01$, for experimental group 2, ZooBioR dose of 5.0 mg/kg fodder).

The obtained results indicated that the administration of the complex biologically active microbial preparation CMP-4 in the daily feed ration of rabbit kits at the rate of 3 or 9 g per 1.0 kg of feed (0.3 or 0.9%/V) for 43 days has increased the productivity (body weight) of animals, has had an anti-stress and hematopoietic stimulating effect, confirmed by haematological blood indices.

CONCLUSIONS

Rabbit farming is a relatively new branch of animal husbandry, and rabbit breeding and exploitation can be successfully carried out under modern animal husbandry conditions, with the application of advanced technologies, which, providing benefits, undeniably also generate stressors with negative impact on the health and welfare of the animals. As a result, the development and testing of new remedies with anti-stress and bio stimulatory properties is becoming a key priority of modern science. In this context, complex undertaken studies have highlighted a new biologically active, environmentally friendly remedy of microbial origin. The elaborated complex has had beneficial effect on the bioproductive parameters of rabbit kits, also, possessed anti-stress and haematopoiesis-stimulating effect in animals, which was confirmed by zootechnical and haematological indices.

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COMPARATIVE RESEARCH BETWEEN PURE BREED KARAKUL AND MEAT CROSSBRED REGARDING THE FREQUENCY OF GENOTYPES AND PREDISPOSITION TO SCRAPIE

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Abstract

The present work aimed to genotyping sheep in order to know the predisposition to scrapie and take the necessary measures. The analyzed biological material was represented by 822 pure breed Karakul sheep and by 239 crossbred meat sheep obtained by crosses between females of the Karakul breed and males of the Palas meat line. The method of determining the genotypes of susceptible sheep to scrapie from biological samples, consists in the analysis of the coding region of the PRNP gene (exon 3) where there are three codons associated with the resistance to this disease. Purebred Karakul sheep which have the genotypes belonging to classes 1 and 2, with the highest resistance to scrapie, have the share of 25.70%, compared to crossbred meat sheep in which the share of genotypes for the mentioned classes was 58.70% (significant for p<0.01 and C.I = 95%). An increase in resistance to scrapie was found in crossbred meat sheep which proves that by practicing infusion crosses with disease-resistant breeds, we will be able to increase scrapie resistance in the case of purebred Karakul sheep.

***Key words**: genotyping, Karakul, resistance, sheep, scrapie.*

INTRODUCTION

The Karakul sheep breed is specialized in the production of skins and has its origin in Asia. In 1910, a total of 160 sheep and rams were imported from Bukhara (Turkmenistan, Kazakhstan). Starting from 1948, the action of crossing the populations of Black Grouse and Greyish from the North-Eastern region of Moldova with Karakul rams was carried out. In this way, the foundations were laid for the formation of a new type of native sheep for the production of hides called Karakul de Botoșani. Currently, there are eight color lines within the breed, respectively Black, Greyish, Brown, Grey, Pink, White, Halili and Sarga. In 1988, it was approved the new sheep breed Karakul of Botoșani with two color lines: Black and Greyish. In 2010 was approved he Brown variety and, in 2018, the Grey one.

The progress registered in recent years in genome analysis technologies has allowed the identification of DNA markers responsible for the variability of phenotypic characters in sheep (Cassmann et al., 2021; McHugh et al., 2022).

The implementation of DNA testing (genotyping) in classical sheep breeding programs based on selection on phenotypic criteria of breeders, can bring important benefits in their genetic management.

The early genotyping of sheep allows for a more efficient matching of mattings, which can have the positive effect of limiting inbreeding, faster fixation of some characters of interest specific to each breed and, last but not least, maximizing genetic progress (Sacchi et al., 2018; Silva et al., 2023).

There are a multitude of genetic diseases that can affect sheep populations and which are of interest for improvement programs. The identification of nucleotide mutations that cause these genetic diseases is essential, because allows the elimination by DNA testing the individuals who carrying mutations associated with undesirable phenotypes.

Scrapie is a lethal contagious disease, which naturally affects mainly the sheep and goats, very rarely other small herbivores. In the manifestation of disease appear characteristic of nervous disorders, caused by the presence of an abnormal prion protein in the nervous system, where it induces specific neurodegenerative lesions, with a spongy appearance. Scrapie is part of a wider group of diseases, called Transmissible Spongiform Encephalopathies - TSEs, with a common element: the appearance of degenerative spongiform lesions in the nervous system. This group of diseases, apart from scrapie, also includes: Bovine spongiform encephalopathy-BSE (mad cow disease), Chronic wasting disease, Mink transmissible encephalopathy, Feline spongiform encephalopathy. There are also several transmissible encephalopathies considered specific to humans: Kuru Disease, Creutzfeldt-Jacob Disease -BCJ, Creutzfeldt-Jacob Disease Variant-vBCJ (Vaccari et al., 2004).

Hence, the need for sheep genotyping to know the predisposition to scrapie and take the necessary measures (Hrincă et al., 2014; Coşier, 2007).

MATERIALS AND METHODS

The biological material analyzed was represented by the active herd of pure breed Karakul 822 sheep and 239 crossbred meat sheep obtained by crosses between females of the Karakul breed and males of the Palas meat line. The entire purebred herd subject to evaluation is included in the Genealogical Register of the breed and is in the improvement program for skin quality.

The rules and responsibilities regarding the method of determining by DNA tests (exon 3 polymorphism analysis of the PRNP gene) the genotypes susceptible/resistant to scrapie from sheep from biological samples (hair, blood and other tissues), consists in the identification of the genotypes susceptible/resistant to scrapie from sheep by analyzing the entire coding region of the PRNP gene (exon 3). In exon 3 are located the three codons (136, 154 and 171) that have been associated in various studies with the resistance or susceptibility of sheep to this disease (Belt et al., 1995; Hunter et al., 1993; Goldmann et al., 1994). Amplification of exon 3 from PRNP gene is carried out from DNA samples purified from blood, hair or other tissues from sheep with the help of specific primers that flank the coding region of this exon and that generate a fragment of over 860 bp. This fragment is then subjected to a sequencing process following which the mutations present in the three codons can be identified based on the generated chromatograms (Coşier & Dărăban, 2016).

The method is applied to identify the sheep with susceptible/resistant genotypes to scrapie using samples of hair, blood or other tissues. Early genotyping of sheep at the PRNP locus allows a rapid selection of genotypes conferring resistance to this disease and subsequently has an improvement in the genetic resistance of native sheep breeds. The presence of the disease was diagnosed in native sheep breeds by histopathological examination (Cătoi et al., 2008) and the frequency of susceptible/resistant genotypes was estimated in various breeds (Coşier, 2007; Coşier et al., 2008; Hrincă et al., 2014; Coşier et al., 2011).

Sample collection is performed under sterile conditions to avoid cross-contamination. In the case of blood samples, they are collected in sterile vacutainers containing K3EDTA as anticoagulant. In the case of hair samples, they must be harvested by plucking (tissues must be harvested with a sterile scalpel) and transferred into sterile plastic tubes. Biological samples must be marked on tubes with identification numbers and will be stored at -200°C until processing in order to avoid their alteration or change in composition.

Reagents required: DNA purification kits, proteinase K, PCR amplification kits (polymerase), agarose, Tris Borate EDTA solution, gel fluorescent dye (Sybr-Safe), DNA length marker (DNA ladder), purification kits, reaction of sequencing, sequencing kits.

Required materials: sample collection tubes, Eppendorf tubes (1.5-2 ml), tips (200 ul, 1000 ul), PCR tubes (0.2 ml), sequencing plates, stands, pipettes, etc.

The required measuring and testing equipment are: DNA sequencing / genotyping laboratory: Genetic analyzer 3130xl Applied Biosystem, Vacuum centrifuge for concentration of nucleic acid samples, centrifuge with cooling, marine bath, electronic balance.

Laboratory for receiving samples, sterilization of solutions and consumables, purification and quantification of nucleic acids: refrigeration unit, chemical hood, centrifuge with cooling, vortex, marine bath, spectrophotometer.

PCR genotyping laboratory (setting reactions / amplification): PCR hood, thermocycler, thermostat, refrigeration unit.

Bioinformatics laboratory: computer, printer.

Electrophoresis laboratory (analysis of amplification products): chemical fume hood, analytical balance, horizontal agarose gel electrophoresis, microwave oven, gel image analysis system.

The genotypes thus obtained can be classified into one of the following classes:

- Risk class 1 (R1): ARR/ARR genotypes - associated with the highest resistance to scrapie;
- Risk class 2 (R2): genotypes ARR/AHQ, ARR/ARH, ARR/ARQ - associated with an average resistance to scrapie;
- Risk class 3 (R3): genotypes AHQ/AHQ, AHQ/ARH, AHQ/ARQ, ARH/ARH, ARH/ARQ, ARQ/ARQ - associated with low resistance to scrapie;
- Risk class 4 (R4): ARR/VRQ genotypes - associated with an increased susceptibility to scrapie;
- Risk class 5 (R5): genotypes ARQ/VRQ, AHQ/VRQ, ARH/VRQ, VRQ/VRQ - highly susceptible to the onset of the disease.

Early genotyping of sheep at the PRNP locus allows rapid selection of genotypes conferring resistance to this disease and therefore allows improvement of the genetic resistance of native sheep breeds.

The data thus obtained, following the genotyping of the flock under study, were systematized and processed statistically. 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 SPSS 16.00 for WINDOWS was used to determine the frequency, the arithmetic mean ($\bar{X}$) the error of the arithmetic mean ($\pm s_{\bar{x}}$) the standard deviation (s), Chi-Square Tests, ANOVA Test, the significance test p. and the confidence interval (BUT.).

The statistical test is a decision method that helps us to validate or invalidate with a certain degree of certainty a statistical hypothesis:

hypothesis H0 (or null hypothesis): the data are not related, they are independent/the compared values do not differ from each other hypothesis H1 (or alternative hypothesis): the data are related, dependent/the compared values differ from each other

RESULTS AND DISCUSSIONS

Regulation (EU) no. 630/2013 of the Commission of June 28, 2013 amending the annexes to Regulation (EC) no. 999/2001 of the European Parliament and of the European Council establishing regulations for the prevention, control and eradication of certain transmissible forms of spongiform encephalopathy, regulates and shows that the objective of the breeding program is to increase the frequency of the ARR allele within the herd, reducing, in at the same time, the prevalence of alleles whose contribution to TSE susceptibility has been demonstrated. The same normative act emphasizes the obligation of individual identification by secure means of the animals in the herd that are to be subjected to a genotyping test.

Therefore, the results obtained from genotyping the active Karakul purebred herd are presented in Table 1.

Table 1. Genotypes of Karakul purebred sheep

Genotip		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	AHQ/ARQ	10	1.2	1.2	1.2
	ARH/ARH	5	.6	.6	1.8
	ARH/ARQ	71	8.6	8.6	10.5
	ARH/VRQ	1	.1	.1	10.6
	ARQ/ARH	1	.1	.1	10.7
	ARQ/ARQ	513	62.4	62.4	73.1
	ARQ/VRQ	11	1.3	1.3	74.5
	ARR/AHQ	1	.1	.1	74.6
	ARR/ARH	11	1.3	1.3	75.9
	ARR/ARQ	180	21.9	21.9	97.8
	ARR/ARR	14	1.7	1.7	99.5
	ARR/VRQ	1	.1	.1	99.6
	Abnormal	3	.4	.4	100.0

Genotip		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	AHQ/ARQ	10	1.2	1.2	1.2
	ARH/ARH	5	.6	.6	1.8
	ARH/ARQ	71	8.6	8.6	10.5
	ARH/VRQ	1	.1	.1	10.6
	ARQ/ARH	1	.1	.1	10.7
	ARQ/ARQ	513	62.4	62.4	73.1
	ARQ/VRQ	11	1.3	1.3	74.5
	ARR/AHQ	1	.1	.1	74.6
	ARR/ARH	11	1.3	1.3	75.9
	ARR/ARQ	180	21.9	21.9	97.8
	ARR/ARR	14	1.7	1.7	99.5
	ARR/VRQ	1	.1	.1	99.6
	Abnormal	3	.4	.4	100.0
	Total	822	100.0	100.0	

Analyzing the information in table 1, we find that sheep with genotypes ARH/VRQ, ARQ/VRQ, ARR/VRQ belonging to classes 4 and 5 with increased and very increased susceptibility to scrapie represent a weight of 1.50%, which represents 13 heads of the total 822 sheep. These sheep have restrictions on sale or breeding and must be slaughtered in 6 months maximum.

Sheep of the pure breed Karakul with genotypes belonging to class 3, associated with low resistance to scrapie have a weight of 72.80% and represent 599 sheep. No sales or breeding restrictions apply to these sheep.

A weight of only 25.70% represents sheep belonging to classes 1 and 2 with the highest and average resistance to scrapie.

Practically, it can be assumed that the classic form of scrapie is spread all over the world at the present time, the only countries considered free of scrapie being Australia and New Zealand, although the disease was reported in them in 1950. According to the O.I.E., if in a country in which epidemiological surveillance is officially practiced and no new cases of scrapie have been reported in the last 7 years, that country can be considered free.

In our country, the first outbreak of scrapie was diagnosed in 2003, within IDSA by Dr. Alexandru Nicolae. Later, other outbreaks were identified in several other counties. The causes underlying the appearance of the disease and its pathogenesis are not completely and definitely elucidated even today. This is because, although it can still be considered an infectious-contagious disease, like most microbial diseases, it clearly differs from all of them in that it is not produced by any bacteria or virus, but by an isomer of a normal protein from the body, without the participation of any nucleic acid (DNA or RNA) to ensure its multiplication. The possibility of transmission of the disease to sheep and goats in natural conditions, as well as experimental transmitssion to other species, were proven long after the description of the disease. The pathogenesis, completely particular, complex and complicated, was partially deciphered later on and as the accumulated results of the studies performed in parallel in the other spongiform encephalopathies, of humans and animals.

The distribution of colors in the Karakul purebred herd taken in the study is shown in Table 2.

Table 2. Absolute and relative frequency for colour at Karakul breed

Colour		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	White	88	10.7	10.7	10.7
	Greyish	80	9.7	9.7	20.4
	Comor	6	0.7	0.7	21.2
	Halili	25	3.0	3.0	24.2
	Brown	77	9.4	9.4	33.6
	Black	257	31.3	31.3	64.8
	Pink	96	11.7	11.7	76.5
	Șarg	1	0.1	0.1	76.6
	Grey	192	23.4	23.4	100.0
	Total	822	100.0	100.0	

The black color in the purebred Karakul has the highest weight of 31.30% and is followed by the yellow color with a weight of 23.40%. The explanation lies in the greater market demand for black skins.

The total score for purebred Karakul sheep is shown in Table 3.

Table 3. Total points at Karakul purebred sheep

Descriptive Statistics	N	Minimum	Maximum	Sum	Mean		Std. Deviation	Variance
	Statistic	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Statistic
Total points	822	390.00	690.00	428417.00	521.1886	1.93579	55.50020	3080.273
Valid N (listwise)	822							

Analyzing the information in the table, we find an average value of 521.18 points with limits between 390 and 690 points. The standard deviation from the mean is 55.50 points.

The results regarding the genotyping of the crossbred meat sheep flock, obtained from crosses between Karakul breed females and Palas meat line males, are presented in Table 4 and Figure 1.

Analyzing the values in Table 4, it can be seen that crossbred meat sheep with genotypes in classes 4 and 5 with increased and very increased susceptibility to scrapie have a weight of 1.20%, and 40.10% represent sheep with genotypes in class 3 associated with an average resistance to scrapie. Correspondingly, classes 1 and 2 with sheep whose genotypes are associated with the highest and average resistance to scrapie have a weight of 58.70% and 140 sheep out of the total of 239 crossbred sheep studied.

So, we see an increase in resistance to scrapie in crossbred meat sheep obtained by crosses between Karakul females and Palas meat line males. From this, we can conclude that by practicing infusion crosses with breeds well analyzed in terms of disease resistance, we will be able to increase the resistance to scrapie in the case of purebred Karakul sheep. Hides as the main production of the Karakul breed must be preserved and preserved, but improvement of milk and meat production is absolutely necessary

Table 4. Genotypes at crossbred meat sheep

Genotypes		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	ARH/ARQ	13	5.4	5.4	5.4
	ARQ/ARQ	83	34.7	34.7	40.2
	ARQ/VRQ	1	0.4	0.4	40.6
	ARR/ARH	6	2.5	2.5	43.1
	ARR/ARQ	105	43.9	43.9	87.0
	ARR/ARR	28	11.9	11.9	98.7
	ARR/VRQ	2	0.8	0.8	99.6
	Genotype	1	0.4	0.4	100.0
	Total	239	100.0	100.0	

Programs to improve the genetic resistance of sheep to scrapie aim to increase the frequency of ARR/ARR type genotypes through genotyping and their promotion in reproduction.

Participation in breeding programs has so far been limited to flocks of sheep of high genetic value. In cases where have been applied, the breeding programs have been effective in increasing resistance to classical scrapie in sheep populations of high genetic value. But the diffusion, within the production populations, of the hereditary factor (allele) which provides the resistance seems to have been, until now, limited. Appendice VII, chapter C of Regulation (EC) no. 999/2001 should allow the genotyping of breeding rams from sheep flocks that are not participating in the breeding program to facilitate a better diffusion of the classical scrapie resistance factor within production populations.

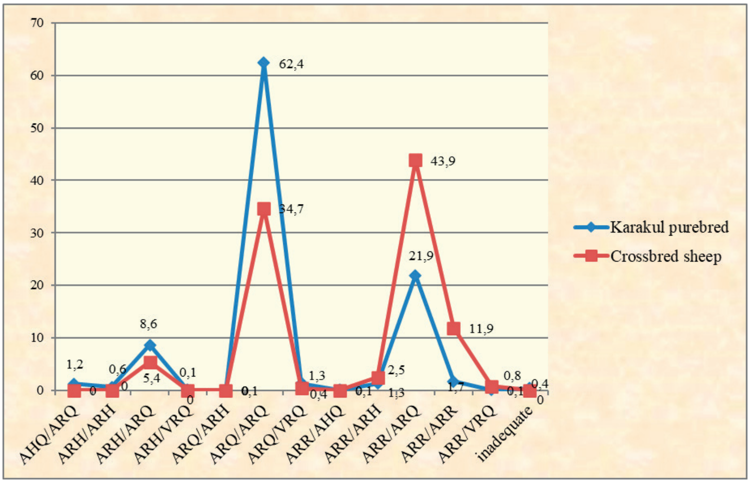

Figure 1. The relative frequency for the genotypes in the studied sheep

From Figure 1, it is evident the increase of the proportion of genotypes with resistance to scrapie in the case of the crossbred sheep for meat studied and the disappearance of some existing genotypes in the Karakul pure breed. The genetic resistance of sheep to transmissible spongiform encephalopathy (scrapie), a disease with a proven hereditary substrate, currently represents one of the main selection criteria of sheep breeders.

CONCLUSIONS

After carrying out the study on the frequency of genotypes and the predisposition to scrapie of sheep of the pure Karakul breed and meat crossbreeds, we can conclude:

1. Sheep of the pure Karakul breed that have the genotypes belonging to classes 1 and 2 with the highest and average resistance to scrapie have the weight of 25.70%, compared to crossbred meat sheep in which the weight of genotypes for the mentioned classes was 58, 70%.
2. In the case of Karakul de Botoșani sheep, it is desirable that scrapie resistance is induced following the selection and use for breeding of individuals with ARR/ARR genotypes.
3. An increase in resistance to scrapie was found in crossbred meat sheep obtained by crosses between Karakul females and Palas meat line males, which proves that by also practicing infusion crosses with disease-resistant breeds, we will be able to increase scrapie resistance.
4. Programs to improve the genetic resistance of sheep to scrapie aim to increase the frequency of ARR/ARR type genotypes through genotyping and their promotion in reproduction.

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RESEARCH ON THE INCIDENCE OF MASTITIS AND ITS INFLUENCE ON MILK PRODUCTION IN A HERD OF CATTLE

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Abstract

The purpose of this work was to study the incidence of mastitis and its influence on quantitative and qualitative milk production. The biological material was represented by two groups of lactating cows: healthy cows (40 heads) and sick cows (9 heads). The obtained data were systematized and processed statistically. In the conducted study, three types of mastitis were highlighted: serous, catarrhal and purulent. The percentage of 18% of cases of mastitis in total population studied, the catarrhal form predominated with 8%, the serous form with 6% and the purulent one with 4%. Average milk production was 4526.05 kg in healthy cows, compared to sick cows in which milk production was 2251.44 kg and the statistically significant difference for p<0.01 it was of 2274.61 kg of milk. Fat and protein content in sick cows case was also reduced, respectively 2.68% for fat and 2.58% for protein. Improving the rearing system, maintaining hygiene in the barn and especially respecting the hygiene of the udder are some of the measures that must be adopted to be able to avoid such unpleasant situations.

Key words: *cows, incidence, mastitis, milk, quantity, quality.*

INTRODUCTION

Inflammatory diseases of the udder occur in cows following microtraumas and/or neglect of hygiene rules during milking. Mastitis is an inflammation of the udder, as a result of a bacterial infection and it can alter a teat, a quarter or the entire udder. The symptoms of the disease are: temperature rise; the appearance of edema; redness of the udder; purulent and bloody discharge of milk from the teats (Berry et al., 2002; Dohoo et al., 2011; Hayes et al., 2001).

There are two types of mastitis in cows: caused by environmental microbes and contagious, caused by Streptococcus agalactiae and Staphylococcus aureus. Both forms cause productive and economic losses. Depending on the manifestation, mastitis is divided into clinical and subclinical form, the latter being symptomless. Detection of mastitis can be done based on external signs and clinical studies. The more highly productive females are, the greater the pressure of infectious agents on the female genital system and the mammary gland (Barkema et al., 1998; Maciuc et al., 2017; Maciuc & Radu-Rusu, 2018; Vidu et al., 2015).

Clinical mastitis can reach an incidence between 13% and 40%, and the economic repercussions can exceed $1000/year in certain countries. Annually, in the United States of America, losses due to cow mastitis exceed 2 billion dollars. At the level of our country, financial losses can reach 200 euros/year/cow with mastitis (Lago et al., 2011a; Lago et al., 2011b; Makovec & Ruegg, 2003; Onaciu et al, 2019; Pantoja et al., 2003).

Ensuring a clean and dry environment is essential for mastitis control because not only milking is a key point but also the times when females can come into contact with moisture, mud and dung so all staff involved in cows care have responsibilities related to reducing the risk of production of mastitis. The evacuation of manure, the type of bedding and keeping the rest areas clean have a major impact on the hygiene of the cows and especially the udders. The study by Barkema et al. (1998) demonstrated that farms where the NCS in the tank is high are deficient in terms of hygiene compared to those where this aspect is well managed. For example, 31% of farms where NCS was greater than 250,000 cells/ml were characterized by a dirty milking parlor compared to 15% of herds where tank NCS was below 150,000 cells/ml. Farms where NCS was greater than 250,000 cells/ml also had more shelters containing more than

10% dung, shelters cleaned less frequently (1.6 vs. 2.2 times per day) and poorer litter use in shelter (Maciuc et al., 2015; Roberson, 2003; Rodrigues et al., 2005; Schutz et al., 2014; Steenveld et al., 2011). Hence, the need for studies on the incidence of mastitis and their influence on milk production in dairy cow farm.

MATERIALS AND METHODS

The analyzed biological material was represented by two groups of cows in lactation wich belonging to the Holstein Friesian breed, respectively from healthy cows (40 heads) and sick cows (9 heads), so 49 heads in total. In our study we considered several objectives such as: the study of the technological flow on the farm, the milking technology on the farm, incidence of diseases on the farm, incidence of mastitis in the analyzed herd, the study of the quantitative and qualitative productive performances according to the health status of cows, the symptomatology, treatment and prevention actions of mastitis in the farm.

The data resulting from observations and direct determinations on the farm as well as from the primary data of the farm, but also from the records of the Official Production Control (COP) carried out by the Cattle Breeders Association, were systematized and statistically processed using the following computer programs: SAVC (Statistics Analysis of Variance and Covariance) respectively SPSS 16.00 for WINDOWS. 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) [3; 8; 10].

For this purpose, the computer program SAVC was used to determine the arithmetic mean ($\overline{X}$), the error of the arithmetic mean ($\pm s_{\bar{x}}$), the standard deviation (s), the Fisher test and Tukey, and SPSS 16.00 for WINDOWS to determine the frequency, Chi-Square Tests, ANOVA Test, Significance test p. and confidence interval (C.I.)

The statistical test is a decision method that helps us to validate or invalidate with a certain degree of certainty a statistical hypothesis:

- hypothesis H_0 (or null hypothesis): the data are not related, they are independent/the compared values do not differ from each other;
- hypothesis H_1 (or alternative hypothesis): the data are related to each other, are dependent/the compared values differ from each other.

The result p of the test, given as a number between 0 and 1, represents the probability of making an error if we reject the hypothesis H_0. If p is lower than the significance threshold α chosen - usually α= 0.05 - we reject the hypothesis H_0 and accept as true the hypothesis H_1 (Cucu et al., 2004; Maciuc et al., 2015; Maciuc & Radu-Rusu, 2018).

The interpretation of p values is done in most statistical tests as follows:

- $p < 0.05$, the relationship is statistically significant (S, 95% confidence);
- $p < 0.01$, the relationship is statistically significant (S, 99% confidence);
- $p < 0.001$, the statistical link is highly significant (HS, confidence 99.9%);
- $p > 0.05$, the relationship is statistically insignificant (NS).

The ANOVA test compares the means of several samples at the same time.

H_0: m1 = m2 = m3 = m4 (for 4 samples)

H_1: at least two means differ significantly

The result is a number p which is interpreted in the same way as the other tests:

- If p>0.05, H0 is not rejected, the difference is insignificant at the 95% significance threshold;
- If p<0.05, H0 is rejected with a significance threshold of 95%. At least two means differ significantly;
- If p<0.01, H0 is rejected with a significance threshold of 99%. At least two means differ significantly;
- If p<0.001, H0 is rejected with a significance threshold of 99.9%. At least two means differ highly significantly.

The Fischer test (F) is used to verify the equality of dispersions of two normally distributed independent variables. The null hypothesis is H_0: σ12=σ22

The Tukey test is the most commonly used multiple comparison procedure, also called the honest significant difference test, usually used in conjunction with ANOVA statistical models.

When the null hypothesis of the F-test in the analysis of variance is rejected, it is of interest to determine what led to this rejection: which means cannot be considered equal. Multiple comparison techniques also appear, because

sequences of comparisons of two means cannot be controlled, as far as the significance threshold is concerned. The Tukey method simultaneously tests all differences between pairs of means to determine if at least one is significantly different from zero.
The Tukey-Kramer test is very similar to the Kramer method for equal groups, but the denominator differs slightly. The formula for calculating Q using Tukey-Kramer is:

$$Q = \frac{M_i - M_j}{\sqrt{MS_{intra} * \left(\frac{\frac{1}{n_i} + \frac{1}{n_j}}{2} \right)}}$$

Where: n_i and n_j represent the number of subjects in the compared groups, and M_i and M_j are the averages of these groups.
The number of degrees of freedom is established similar to the Tukey method. The first degree of freedom is k (the number of groups of the experiment) and the second is N-k (df for intragroup dispersion).

RESULTS AND DISCUSSIONS

The exploitation system of cows in the studied farm is that of free stables one, with the capacity to raise 50 dairy cows (Figure 1).
The cow shelter has the appearance of the letter "L", extended over 526 m^2. The height regime of the shelter is of the "ground floor" type, with dimensions of 32.87 x 14.03 m in plan, in length and 9.75 x 6.65 m, in width. This positioning allows ensuring a favorable microclimate for housing and exploitation of dairy cows.
The floor is made of road concrete, with a thickness of 15 cm and of ballast with a thickness of 10 cm. Inside, the stands are arranged frontally in two rows (Figure 1) and are 2.20 m long and 1.2 m wide.
The stands have a floor made of rubber carpets, 2 cm thick, placed directly on the reinforced concrete. The rubber bedding had superficial striations with an anti-slip role, which ensure a complete draining of liquids (under and above the bedding). The floor of the stand has a drainage slope in the first two thirds of 1% and in the last third of 2%.
The movement alley is located in front of the feed front and between the rows of resting beds, it is 3.00 m wide and has a drainage slope (2%) for liquid manure. The alley has margins that keep vehicles on the traffic side. The feeding front is individualized and equipped with a continuous metal frame, set back 10-20 cm from the edge of the manger. It presents pillars at intervals of one or two stands, reinforced by stand separating bars.

Figure 1 The livelihood system applied in the studied farm (original photo)

The feed lane is used by the farmer to distribute forage. It is 4 m wide, and the floor is made of reinforced concrete (10 cm thick), with a wear layer of rolled cement (2 cm thick). At a height of 10-15 cm, between the floor and the wall plinths are made.
The manure is removed with a scraper plow that works in the area of their movement.
The farm has a fodder park on an area of 110.01 m^2 which is supplied with bales of alfalfa and of hay and with corn silos. Feeding of the cows is done with a technological trailer.
The milking parlor is equipped with a fish-type installation (2 x 4), located near the dairy, a space intended for taking over and cooling the milk. From here, the quantitative and qualitative evaluation of the milk is done, then it is delivered to the processing company.
The cases of diseases found in the farm under study were mastitis and laminitis.
From the group of mastitis, the most frequent were:
- serous mastitis manifested by swelling of the affected part of the udder; low productive level; the milk from the affected quarter has a more liquid consistency and often changed in color. The local temperature is increased and after

milking the edema decreases. The lymph nodes are enlarged;

- catarrhal mastitis in which the general condition of the animal is normal. Production drops insignificantly. If the catarrh persists in the galactophorous ducts, casein clots are observed in the milk at the beginning of milking. If the glandular acini (alveoli) are affected, clots appear at the end of milking. The local temperature is increased. On palpation at the base of the teats it can be detected induration of the size of a pea;
- purulent mastitis is manifested by the depressed condition of the cow; limps and does not chew the cud. Body temperature exceeds 40°C. There is no milk in the affected quarter. The lymph nodes are enlarged.

From the pododermatitis group, the following persisted:

- aseptic pododermatitis manifested by serous, serous-hemorrhagic or serous-fibrous inflammation of the hoof skin;
- purulent pododermatitis is a purulent inflammatory process of the base of the hoof skin of an individual. It develops as a complication after aseptic pododermatitis and also occurs with cracks, wounds, folds of the horn of the hoof wall.

Table 1 and Figure 2 show the cases of disease found on the farm, the incidence of diseases and mastitis in the herd of cows studied and we note that the percentage of healthy cows in herd was only 53%.

From the laminitis group, the aseptic form represented 17% of the total followed by the purulent form 12%. From the group of mastitis, the catarrhal form predominated, with 8%, then the serous form, with 6% and the purulent form, with 4%.

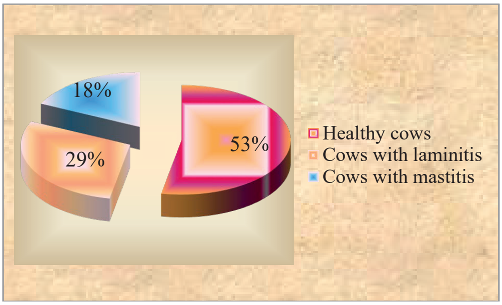

Figure 2. Graphical representation of diseases in the studied herd

Table 1. Incidence of diseases in the farm

Total herd (head)	Healthy cows		Cows with laminitis				Cows with mastitis					
	heads	%	aseptic		purulent		serous		catarrhal		purulent	
			head	%	head	%	head	%	head	%	head	%
49	26	53	8	17	6	12	3	6	4	8	2	4

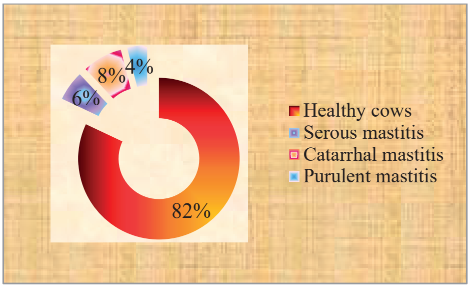

Figure 3 The incidence of mastitis in the studied farm

In the studied farm, three types of mastitis were encountered: serous, catarrhal and purulent. From Table 2 and Figure 3 it is highlighted that from total herd, 18% are cases of mastitis, of which the catarrhal form predominated, with 8%, then the serous form, with 6% and the purulent one, with 4%.

Table 2. The incidence of mastitis in the studied farm

Total herd (heads)	Healthy cows		Cows with mastitis					
	heads	%	serous		catarrhal		purulent	
			heads	%	heads	%	heads	%
49	40	82	3	6	4	8	2	4

The productive level for the groups of healthy and sick cows was analyzed during a single lactation (total and normal), the mean values of the analyzed indicators being presented in

Tables 3 and 4. The monitored characters were: the duration of lactation (days), the amount of milk (kg) age of first calving (months) and service period (days). Regarding the milk quality, two characters were analyzed, fat and protein (percentage and quantity). The duration of lactation character recorded in first total lactation, for healthy cows, an average of 473.48 days with variations between 366 days and a maximum of 713 days. For the group of sick cows, the duration of lactation was on average 268.44 days, with variations between 195 days and 328 days, significantly reduced lactation for $p< 0.01$, C.I. = 95%, compared to healthy cows. In normal first lactation, the group of healthy cows recorded an average duration of lactation of 304.65 days, but in sick cows, the mean was only 265.33 days, with variations between 195 and 305 days.

Table 3. Statistics of milk production in the herd studied with healthy animals

Lactation	Traits	n	$\overline{X}$	$\pm s_{\bar{x}}$	s	V%	Minim	Maxim
First total lactation	Length of lactation (days)	40	473.48	12.44	78.70	16.62	366	713
	Milk production (kg)	40	5766.18	192.63	1118.29	19.12	3375	7919
	Fat, %	40	4.05	0.04	0.29	7.37	3.38	4.71
	Fat (kg)	40	234.23	8.65	54.72	23.36	114.08	345.2
	Protein, %	40	3.63	0.02	0.12	3.51	3.45	4.00
	Protein (kg)	40	209.48	7.16	45.28	19.62	122.18	306.78
First standard lactation	Length of lactation (days)	40	304.65	0.17	1.12	0.36	300	305.00
	Milk production (kg)	40	4526.05	153.15	968.64	19.40	1879	8207
	Fat, %	40	4.06	0.05	0.32	7.91	3.6	4.9
	Fat (kg)	40	183.77	6.94	43.88	19.88	80.8	344.69
	Protein, %	40	3.17	0.02	0.17	5.42	3.00	3.83
	Protein (kg)	40	142.98	4.65	29.44	19.59	63.89	251.95
	A.F.C. (months)	40	26.43	0.19	1.24	4.68	23.55	29.00
	S.P. (days)	40	68.7	2.18	13.81	20.10	25.00	89.00

AFC: age of the first calving; SP: service period

Table 4. Statistics of milk production in the herd studied with sick animals

Lactation	Traits	n	$\overline{X}$	$\pm s_{\bar{x}}$	s	V%	Min	Max
First total lactation	Length of lactation (days)	9	268.44	16.66	50.00	18.62	195	328
	Milk production (kg)	9	2331.89	100.80	302.40	12.96	1865	2684
	Fat, %	9	2.82	0.10	0.31	11.31	2.33	3.28
	Fat (kg)	9	65.74	4.02	12.06	18.35	47.86	88.04
	Protein, %	9	2.55	0.14	0.43	16.94	2.04	3.3
	Protein (kg)	9	60.72	5.24	15.73	21.91	41.96	87.48
First standard lactation	Length of lactation (days)	9	265.33	15.50	46.51	17.53	195	305
	Milk production (kg)	9	2251.44	159.47	478.42	17.25	1604	2956
	Fat, %	9	2.68	0.07	0.23	8.79	2.33	2.98
	Fat (kg)	9	59.99	4.27	12.82	19.38	44.27	80.58
	Protein, %	9	2.59	0.16	0.50	19.53	2.04	3.28
	Protein (kg)	9	57.62	4.93	14.81	21.70	36.89	80.85
	A.F.C. (months)	9	26	0.38	1.16	4.46	23.99	27.5
	S.P. (days)	9	105.22	1.70	5.11	4.86	96	110

The average production of milk per total lactation for the group of healthy cows (Table 3) was 5766.18 kg of milk in the first total lactation (variations being between 3375 and 7919 kg), and for the group of sick cows (Table 4), the average production of milk was 2331.89 kg in first total lactation and 2251.44 kg of milk in first standard lactation (305 days) with variations between 1604 kg and 2956 kg. The variability of the milk quantity character, for both groups of cows, healthy and sick, had average values (17-19%).

Statistically, the differences in the mean values for the milk quantity trait in the Fisher test was very significant, and the Tukey Test shows us a very significant difference of the means between the two lots of 2274.61 kg of milk (C.I = 95%).

Regarding the fat percentage and protein percentage traits for the group of healthy cows, the average values were 4.05-4.06% for fat % and 3.63-3.17%, for protein %. In the group with sick cows, the average of traits had values between 2.68 - 2.82%, and for proteins, the average values were within the limits of 2.55-2.59%.

About the quantity of proteins, the mean of the group with healthy cows was between 142.98-209.48 kg, compared to the mean values of 57.62-60.72 kg for sick cows.

In parallel with the analysis of the productive level, emphasis was also placed on two reproductive indicators, namely the age of the first calving (AFC) and the service period (SP). Thus, in the group of healthy cows, the mean of AFC was 26.43 months (with variations between 23.55 and 29 months), compared to 26.00 months (with variations between 23.99 and 27.5 months), in sick cows.

The duration of the SP was 68.7 days in healthy cows (with variations between 25 and 89 days), compared to 105.22 days in sick cows, with variations between 96 and 110 days.

In Tables 5 and 6 we present the main indicators for milk quality in the two batches of cows studied, respectively healthy cows and sick cows.

Table 5. Statistics for qualitative indicators of milk production in healthy cows

Traits	n	$\overline{X}$	$\pm s_{\bar{x}}$	s	V%	Minim	Maxim
NSC (ml x 1000)	40	286.65	10.977	69.426	24.22	140	430
Fat, %	40	3.94	0.012	0.075	2.157	3.37	3.84
Protein, %	40	3.38	0.015	0.095	2.809	3.25	3.61
Lactose, %	40	4.11	0.038	0.241	5.874	3.88	4.58
SUT, %	40	11.93	0.233	1.472	15.293	9.9	14.3
Urea (mg/dl)	40	13.5	0.184	1.166	8.638	12.25	16
Caseink, %	40	29.82	0.22	1.389	4.659	24.37	32.3
Daily milk production (kg)	40	14.57	0.268	1.693	11.617	10.8	18.3

Table 6. Statistics for qualitative indicators of milk production in sick cows

Traits	n	$\overline{X}$	$\pm s_{\bar{x}}$	s	V%	Minim	Maxim
NSC (ml x 1000)	9	568.44	38.738	116.214	20.444	420	793
Fat, %	9	3.45	0.091	0.274	8.699	2.76	3.65
Protein, %	9	2.91	0.123	0.368	12.627	2.3	3.28
Lactose, %	9	4.53	0.046	0.137	3.012	4.26	4.7
SUT, %	9	9.98	0.019	0.057	0.638	8.93	10.06
Urea (mg/dl)	9	10.88	0.224	0.671	6.165	10	11.86
Casein, %	9	26.78	0.156	0.468	1.628	28.1	29.3
Daily milk production (kg)	9	7.88	0.467	1.401	17.773	5.65	9.58

From the tables with the centralized results, we find that the mean value of the somatic cell count, the indicator that indicates the health of the udder and the quality of the milk, was 286.65 ml x 1000 in healthy cows compared to 568.44 ml x 1000 in sick cows, the difference between the two groups being highly significant for $p < 0.01$ and CI = 95%. Significant differences for p

< 0.05, C.I. = 95%, we also found in the fat and protein content of the milk, in the two groups of cows studied. Accordingly, I found at SUT respectively the casein from milk for healthy and sick cows.

In Figures 4 and 5 it is present the regression line for protein and lactose, respectively the number of somatic cells (NSC) and milk casein.

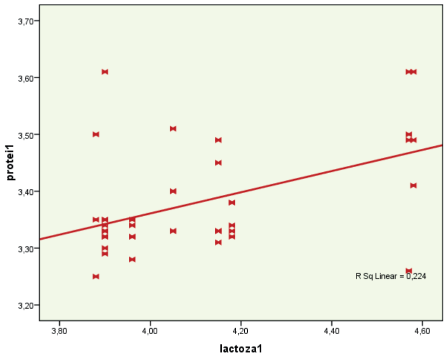

Figure 4. Regression line for protein and lactose

The upward evolution of the regression line indicates a positive evolution of the analyzed indicators, with a low intensity of 20-22% (0.20-0.22). A high protein content in milk will cause more lactose in milk and vice versa. Lactose is a type of natural sugar found in milk and milk products and it is a disaccharide consisting of two sugar molecules - glucose and galactose - which are linked by a beta-glycosidic bond.

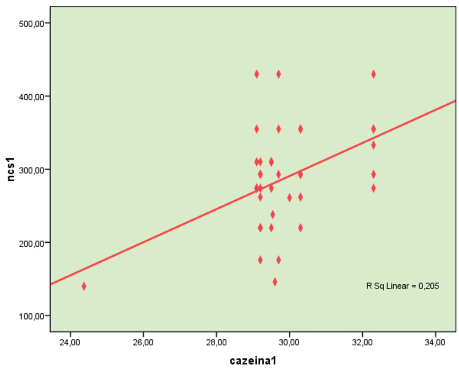

Figure 5. Regression line for NSC and casein

Casein is a complex protein found in milk. This is the protein with the highest presence in milk, representing 80% of the total proteins in its composition. Problems arise when for unknown reasons the immune system identifies casein as harmful. Thus, when it "identifies" the presence of casein in the body, the immune system activates specific antibodies, type E immunoglobulins or IgE, releasing histamine, a substance that causes tissue inflammation.

In Figure 4 we find that the number of somatic cells influences the casein content of milk by 0.20 or 20%. The evolution of the regression line is upward, and the connection between the two studied indicators is positive.

CONCLUSIONS

Following the study on the incidence of diseases and mastitis in a herd of cows and the influence on milk production, we can conclude:

1. Diseases and mastitis influence milk production both quantitatively and qualitatively, causing great problems in dairy farms. The EU regulations do not exclude from processing and consumption the milk of subclinical udders but the abnormal milk detected during the individual test milking done by the milker and the non-compliant milk (the merged one), i.e. the one whose NSC and NTG exceed 400,000/ml and 100,000/ml respectively, because it includes a large percentage of milk with mastitis.
2. Dry period allows the intramammary epithelium to regenerate so that when lactation begins again, milk production is optimal. It is required for the udder a rest period of at least 40 days to achieve optimal milk production. Therapy during the dry period reduces the risk of new "environmental" infections, especially in cows with a history of mastitis or high NSC.
3. Ensuring a optimal microclimate in the barn and in the pasture, a clean and dry bedding that limits the grafting of germs on the teats, correct milking regardless of the rearing system, the application of prophylactic procedures before and after milking, the isolation or exclusion of females with forms severe of mastitis, periodic screening of milking parlor parameters, periodic screening of milk quality, the application of targeted therapies on the pathogenic germ causing the disease, represent the main measures that must be adopted in dairy farms to prevent the spread of mastitis and the problems that arise due to this disease.

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MORPHOSTRUCTURAL CHARACTERIZATION OF *Longissimus dorsi* MUSCLE TISSUE OF AUBRAC CATTLE

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Abstract

The purpose of this paper was to highlight the results of the morphostructural characterization of Longissimus dorsi muscle tissue of the Aubrac breed, exploited in Romania. The present research focused on a population of 38 cattle, including both males and females, raised in a semi-intensive farming system. Longitudinal measurements (major and minor diameter, mean, DM/Dm ratio, length) as well as transverse measurements (diameter, area) of muscle fibres were conducted, and the results were statistically interpreted. In terms of determining the length of muscle fibres in the longitudinal section, significant differences between genders are observed. In males, the large diameter recorded values of 77.17 µm, while in females, it was 64.73 µm. Regarding the determination of the area of muscle fibres in cross-sectional analysis, significant differences were noted between males and females (in males, it`s an average of 2226.83 µm$^2$, while in females it`s 1576.60 µm^2). This shows that in the Aubrac breed, the area of muscle fibres in meat content can vary by gender. In conclusion, longissimus dorsi muscle is particularly important and valuable in the beef industry.

***Key words**: beef cattle, Longissimus dorsi, morphostructure, quality.*

INTRODUCTION

One of the oldest cattle breeds in France is the Aubrac cattle breed, originating from the Aubrac mountains in the southern Massif Central. Initially, the cattle were raised as a dual-purpose breed, as their milk was used in the production of Laguiole cheese. Over time, particularly after the post-war period, the Aubrac breed has been predominantly raised for meat production. Its adaptable characteristics and efficient growth qualities have made Aubrac a popular choice for the meat industry. Representatives of the Aubrac breed generally have a medium build. Adult cows weigh between 550 and 800 kg and stand 130 cm tall, while bulls stand 140 cm tall and weigh between 900 and 1200 kg (Madescu et al., 2022).

Aubrac cattle are easy to maintain as they efficiently consume any type of feed. They quickly adapt to grazing on large, resource-poor pastures without significantly impacting their productivity. The Aubrac breed is renowned for the exceptional quality of its meat, with distinctive flavors and tenderness. A high level of marbling highlights the distinct flavor and delicate texture of meat from the Aubrac breed (Madescu et al., 2021). The breed, age, sex, and health status of the animals, as well as the methods of breeding, feeding, slaughtering, and processing, all have an impact on the quality of beef. One of the most important factors is marbling, which refers to the even distribution of fat within the muscle tissue and is essential for a fine texture and rich flavor. Additionally, the color, texture, and taste of the meat are key aspects of assessing quality. Animals raised naturally and grass-fed without additives, as well as those from responsible farming systems, can produce high-quality meat (Vidu et al., 2015). Certifications and standards that attest to the conditions of breeding and processing can also be important indicators of beef quality. Overall, superior-quality beef is valued for its distinctive aroma, succulent texture, and rich taste. The term "meat" refers to the muscular tissue of the slaughtered animal, together with the tissues naturally connected to it: fat, bones, tendons, aponeuroses, connective tissue, blood vessels, nerves, lymph nodes, by-products, and organs.

From a morphological standpoint, meat comprises: muscle tissue, striated; connective tissue; adipose tissue; bone tissue; blood vessels; and the proportion of various tissues that make up meat depends on the animal's breed, age, sex, and fattening state (Sadeghinezhad et al., 2016). The average proportion of components in bovine meat is: 58% muscle tissue, 18% bones, 12% fat, and 12% connective tissue with blood vessels and nerves. Therefore, it is evident that muscle tissue, which also makes up the majority of the animal's body, represents the majority of meat.

Connective tissue represents the second morphological component of meat. It is found in all segments of the animal's body and is a determining factor in the quantity and quality of meat (Stimbirys et al., 2016). The proportion of this tissue influences characteristics such as tenderness, color, water loss capacity through boiling, meat juiciness, and marbling finesse. The structure of adipose tissue. This represents a specialized form of connective tissue. The third tissue is adipose tissue, which is composed of fat cells covered with a protoplasmic membrane containing the nucleus (Fabbri et al., 2021). The basic morphological unit of adipose tissue is the adipose cell of fibrocellular origin.

The longissimus dorsi muscle is a large and elongated muscle located on the lateral side of the vertebral column in cattle. It extends along the thoracic and lumbar regions, starting from the cervical region to the base of the tail (Choat et al., 2006). This muscle is responsible for the extension, flexion, and rotation of the vertebral column in animals. Additionally, it acts as a stabilizer of the vertebral column and plays an important role in trunk movements.

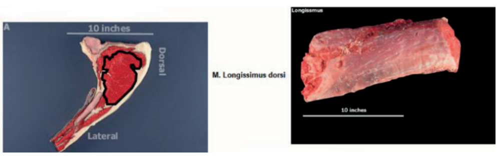

Figure 1. *Longissimus dorsi* muscle (processed photograph)

The *Longissimus dorsi* muscle is one of the most valuable muscles in the beef industry. It is prized for its organoleptic qualities, such as its tender texture and rich flavor (Fabbri et al., 2021). From this muscle, some of the most popular and esteemed beef cuts are obtained, such as the ribeye, the middle loin (T-bone, porterhouse), the New York strip steak, and the striploin roast (Choat et al., 2006). The histological structure of muscles is highly relevant, both theoretically and practically, regarding meat quality. It influences a range of physical, chemical, and technological properties of meat, significantly impacting its texture, juiciness, taste, and overall quality. An important aspect of histological structure is the fineness and density of muscle fibers. Thin and dense muscle fibers are associated with tender and visually appealing meat, as they allow for easy cutting and provide a more uniform texture. Conversely, thicker and less dense muscle fibers can result in tougher and less tender meat (Dransfield et al, 2003).

Additionally, the size of muscle bundles and the proportion of muscle and connective tissue have a significant impact on meat characteristics. Larger muscle bundles can influence the appearance of meat and may be associated with more voluminous meat. At the same time, the optimal proportion of muscle tissue to connective tissue can contribute to juicier and more flavorful meat (Foggi et al., 2021).

All these aspects related to the histological structure of somatic muscles are essential in determining the quality of Aubrac beef. By understanding and monitoring these characteristics, high-quality meat with pleasant texture, juiciness, and excellent taste can be achieved, thus meeting consumer requirements (Chriki et al., 2013).

MATERIALS AND METHODS

The study involved examining a total of 38 cattle, comprising both males and females, with ages ranging from 15 to 18 months, which were reared under a semi-intensive farming system. After slaughter, samples were collected from the carcasses obtained from Aubrac breed animals, and the morphostructure of the *Longissimus dorsi* muscle was analyzed. Subsequently, for interpreting and understanding the results, the SPSS (Statistical Package for the Social Sciences) analysis software was used, applying statistical methods such as ANOVA (Analysis of Variance) and the Tukey Test.

Histological technique - The dimensions of the specimen were approximately 2 cm in length and 1 cm in height, to allow for rapid penetration of the fixative throughout the sample (Figure 2). After a fixation period of at least 24-48 hours, the specimens will be sectioned with sharp blades to obtain a much thinner thickness, and then they will be placed into fresh fixative.

Figure 2. Cross-sectional view of Aubrac cattle beef (original photo)

The embedding in paraffin occurred in four successive phases: dehydration, clarification, impregnation with paraffin, and actual embedding. Histological sectioning of the paraffin was performed using a microtome, which provided the opportunity to obtain sections with a thickness of 5-6 μm. The sections were stained using the Hematoxylin-Eosin-Methyl Blue (HEM) method.

Figure 3. Stages of processing and obtaining histological slides (original photo)

After obtaining the desired slides, they were subjected to microscopic analysis, where longitudinal measurements (major diameter, minor diameter, mean diameter, DM/Dm ratio, length) and transverse measurements (diameter, area) of the muscle fibers were performed (Figure 3).

The Leica 1CC50w microscope is a model used for histological analysis. It utilizes transmitted light to visualize the structure and composition of tissues at a microscopic level. It is equipped with multiple objectives for various magnification levels and has an adjustable illumination source for optimal contrast (Figure 4).

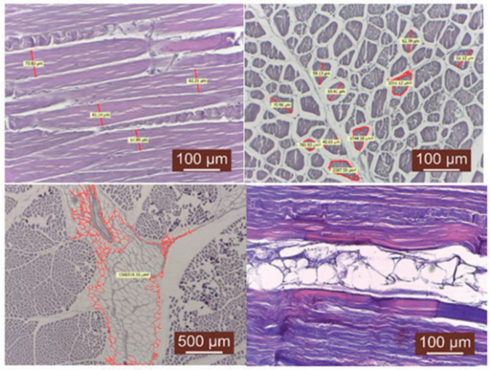

Figure 4. Analysis of histological slides under the microscope (original photo)

Additionally, it features precise focusing mechanisms and binocular eyepieces and tubes for simultaneous observation or stereoscopic visualization.

Subsequently, the obtained results underwent rigorous statistical analysis to be interpreted in detail. This involved the use of appropriate statistical methods to assess the significance and correlations between the obtained results. Various techniques were employed to extract relevant information from the data obtained. The statistical interpretation of the results provided a deeper understanding of the differences and relationships within the dataset, allowing for the formulation of conclusions and generalizations regarding the study parameters. This process of statistical interpretation provided a solid foundation for understanding and interpreting the results obtained in the present research.

RESULTS AND DISCUSSIONS

Longitudinal measurements (major diameter, minor diameter, mean diameter, DM/Dm ratio, length) as well as transverse measurements (diameter, area) of the muscle fiber were conducted. Table 1 highlights the mean values obtained within the *Longissimus dorsi* muscle, categorized by sex, following the muscle analysis.

Table 1. Morphostructure of the *Longissimus dorsi*, Psoas, Semitendinosus, and Deltoid muscles

Specification (μm)	Gender	Muscle region M. Longissimus Dorsi		
		$\bar{X}$±SD	Min.	Max.
*Major diameter (DM)	M	77.17 ± 5.34^{xA}	69.30	83.67
	F	64.73 ± 4.63^{yC}	57.42	72.77
*Minor diameter (Dm)	M	63.37 ± 3.22^{xA}	59.31	68.51
	F	53.05 ± 2.13^{yC}	49.22	55.97
* Mean diameter (D$\bar{x}$)	M	70.27 ± 2.47^{xA}	66.65	75.07
	F	58.89 ± 2.47^{yB}	53.82	63.00
*DM/Dm ratio	M	1.22 ± 0.12^{xB}	1.02	1.38
	F	1.22 ± 0.10^{xB}	1.04	1.41
*Length	M	558.77 ± 19.79^{yA}	540.20	590.20
	F	587.68 ± 12.91^{xA}	562.00	608.00
**Mean diameter	M	71.11 ± 2.65^{xA}	65.88	74.31
	F	59.56 ± 3.53^{yC}	53.34	64.57
**Cross -section area (μm²)	M	2226.83 ± 233.15^{xA}	1769.34	2574.93
	F	1576.60 ± 310.37^{yB}	1116.42	2006.31

Note: *Longitudinal section of muscle fiber; **Transversal section of muscle fiber; x & y: There are no significant differences (P > 0.05) between any two means within the same column with the same letter index; A, B, and C: There are no significant differences (P > 0.05) between any two means within the same row with the same letter index; M – males, F – females.

In the course of the conducted research, significant differences were observed between the average diameter of muscle fibers in males compared to that recorded in females, in the case of M. *Longissimus dorsi*. This indicates that, in the Aubrac breed, gender can influence the thickness of muscle fibers, with an average value of 70.27 ± 2.47 μm in males, while in females, it is lower, at 58.89 ± 2.47 μm, showing significant differences between sexes.

Additionally, for instance, significant differences are noted between sexes regarding the ratio of the large diameter to the small diameter, with males recording an average value of 1.22 ± 0.12 μm, while females have a value of 1.22 ± 0.10.

Regarding the determination of muscle fiber length in longitudinal sections, significant differences between sexes are observed, with males having an average value of 558.77 ± 19.79 μm and females having an average value of 587.68 ± 12.91 μm. This shows that, in the Aubrac breed gender can influence the length of muscle fibers. By examining the longitudinal sections of muscles, valuable information can be obtained about the distribution, density, and size of muscle fibers, as well as the proportion of muscle tissue and connective tissue (Foggi et al., 2021). These aspects are essential in determining the quality of meat, as they influence its texture, juiciness, and tenderness.

Moreover, within the conducted research, activities also focused on determining the mean diameter and area of the cross-sectional area of the muscle fiber in both sexes.

On the cross-sectional section, significant differences are observed between the mean diameter of muscle fibers in males (71.11 ± 2.65 μm) compared to that recorded in females (59.56 ± 3.53 μm), in the case of the *Longissimus dorsi* muscle.

These findings indicate that, in the Aubrac beef cattle breed, gender may have implications for the mean diameter of muscle fibers within the region under study.

The mean diameter of muscle fibers is an important measure of muscle structure in beef cattle bred for meat production. This parameter indicates the average thickness of muscle fibers within a particular muscle and can influence the quality and characteristics of the meat. On the other hand, a smaller diameter of muscle fibers may be associated with more tender and delicate meat, as these fibers can be softer and less dense. This may be desirable for certain types of meat, such as high-quality beef, which is valued for its fine texture and succulence.

Regarding the determination of muscle fiber area in cross-section, significant differences are observed in males (2226.83 ± 233.15 µm) compared to those recorded in females (1576.60 ± 310.37 µm), in the case of M. *Longissimus dorsi*. This indicates that, in the Aubrac breed, gender can influence the muscle fiber area in the meat content.

The term "muscle fiber area" refers to the entire surface that a particular muscle's muscle fibers cover, and it can reveal details about the density and distribution of muscle fibers within the muscle tissue. A larger area of muscle fibers may indicate better muscle development and a higher fiber density. This can contribute to better-marbled and more muscular beef (Sadeghinezhad et al., 2016). Higher muscle fiber density can also influence the texture and juiciness of the meat, providing a more enjoyable chewing sensation and a richer taste. On the other hand, a smaller area of muscle fibers may be associated with a finer and more delicate meat structure. This can create an easier and less resistant chewing sensation during consumption.

In the conducted research, in order to provide a more concrete understanding of the morphostructure of the meat derived from the Aubrac cattle breed, along with the analysis of the samples under the microscope, the adipose tissue content was also examined. The adipose tissue within the structure of the *Longissimus dorsi* muscle in the Aubrac cattle breed (Figure 5) represents an important component that influences the characteristics of the meat, such as appearance, texture, and taste.

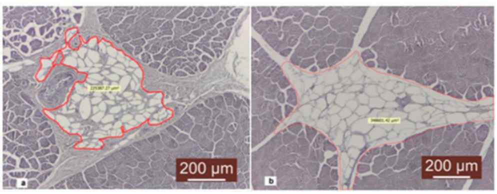

Figure 5. Highlighting of adipose tissue from the *Longisimus dorsi* muscle (a - male; b - female)

The presence of intramuscular fat in the *Longissimus dorsi* muscle contributes to the tenderness and juiciness of the meat. The quantity and distribution of fat within the muscle can vary depending on several factors, including the age, diet, and level of activity of the animal (Haderlie et al., 2023). From the obtained images, we can observe that Aubrac cattle exhibit remarkable marbling in the Longissimus dorsi muscle, which can impart the meat with a distinct texture and juiciness.

CONCLUSIONS

In conclusion, based on the morphostructural analysis of the meat, significant differences were found between sexes regarding the diameter and length of muscle fibers, as well as the mean diameter and area of muscle fibers. These differences can be influenced by several factors, including the hormonal composition and genetic characteristics of the animals. In general, beef muscle fibers from males (bulls) may be larger and thicker than those from females. This is due to the higher production of anabolic hormones such as testosterone in males. Testosterone stimulates muscle growth and hypertrophy of muscle fibers, leading to more developed and thicker muscle fibers in bulls. On the other hand, beef from females may have a higher amount of intermuscular adipose tissue. This can influence the texture and taste of the meat, giving it greater succulence and tenderness compared to that from males. Obtaining these results contributes to the advancement of scientific knowledge and a deeper understanding of the characteristics of meat from Aubrac cattle. This information can be used in further research to explore aspects related to nutrition, genetics, and processing technologies that could enhance the quality and value of meat from Aubrac cattle.

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MICROBIOLOGICAL AND HYGIENIC QUALITY OF AUBRAC CATTLE FRESH MEAT

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Abstract

This work aimed to investigate and evaluate the microbiological control of carcasses from Aubrac cattle, focusing on ensuring quality and food safety in the meat industry. In this research, three microbiological parameters were monitored: contamination with Salmonella, total bacterial count and contamination with Enterobacteriaceae. The sponge sampling method was employed, involving the wiping of a 100 cm^2 surface area with a template using sponges soaked in sterile diluted peptone. Sampling was carried out randomly from ten carcasses (males and females), selecting four areas with the highest contamination frequency from each carcass, resulting in a total surface area of 400 mm^2. Following the tests, it was seen that the samples did not contain any bacteria from the Salmonella genus or the Enterobacteriaceae family. In terms of the total number of bacteria, the highest microbial load was found in males (8.2 x 102 cfu/cm^2) on carcasses 2 and 5. Simultaneously, the lowest microbial load (6.0 x 102 cfu/cm^2) was recorded on carcass 4 from females. We can conclude that, based on the results, these values are below the accepted standard limit, indicating wholesome meat.

***Key words**: beef carcass, food safety, microbiology.*

INTRODUCTION

The Aubrac breed is a cattle breed originating from the Aubrac region in southern France. It is known for its resilience and adaptability to environmental conditions, adapting very well to the pedo-climatic conditions in Romania (Madescu et al., 2021). The meat from Aubrac cattle is appreciated for its superior quality, with a fine texture and delicate flavor. It is considered high-quality meat due to its tenderness and high marbling content, which contribute to its juiciness and flavor (Bakharev et al., 2017).

The quality of food products has a much broader significance than that of other products, as it has much deeper effects since it is fundamental to life, determines the course of metabolic processes, and can influence the development of the entire organism. Microbial contamination of carcasses occurs as a result of inadequate hygiene conditions during slaughter, processing, and meat handling. Contamination can be reduced through good processing practices, but the complete elimination of pathogenic germs is nearly impossible. Numerous techniques have been developed to reduce contaminating bacteria on carcass surfaces, but most current procedures involve washing and disinfection.

The meat intended for consumption must come from healthy animals, as some diseases can be transmitted to humans through ingesting contaminated pieces (Clinquart et al., 2022). Due to its chemical composition, especially its highwater content, meat provides a conducive environment for the growth of microorganisms. Risks associated with consuming fresh meat include infections caused by certain bacteria, such as Salmonella, Lister, and Enterobacteriaceae, as well as the presence of parasites (Atsbha et al., 2018).

Microorganisms are extremely small, making it impossible to observe them with the naked eye during post-mortem inspection. However, visual inspection of the meat can detect lesions, fecal contamination, and various foreign bodies that may constitute contamination (Tesson et

al., 2020). To specifically identify microorganisms in meat, specific laboratory techniques are required (Fegan et al., 2004).
In any Hazard Analysis and Critical Control Point (HACCP) program for primary meat processing, the condition of the animal represents a critical control point. The physiological state of the animal and the internal and external microbial load are important factors for the final microbiological quality of the meat after slaughter (Manyori et al., 2017). This does not mean that other factors, such as facility design, slaughter procedures, and adherence to good practice standards, are not important in ensuring the production of hygienic and high-quality carcasses.
Generally, it is assumed that preventing visible contamination of carcasses will enhance the microbiological safety of the meat (Warmate et al., 2023).Visible carcass contamination can be reduced by washing, skinning before flaying, and paying increased attention to evisceration and flaying so as not to transfer dirt to the meat (Gonzales-Barrón et al., 2016).It would be preferable to control visible contamination through superior flaying and evisceration practices rather than by applying washing treatments that may not always promote the removal of a substantial number of bacteria from carcasses. Washing reduces visible contamination and does not affect the microbiological condition of the carcass, but in the worst case, due to humidity, bacteria can multiply more easily (Brown et al., 2013).
Determining the microbiological parameters of Aubrac beef has multiple benefits. This information can be used to assess and improve quality, monitor production and hygiene processes, ensure food safety, and meet consumer requirements and preferences. Additionally, these evaluations can contribute to promoting and valorizing high-quality meat and developing a more sustainable food industry (Cummins et al., 2016).

MATERIALS AND METHODS

For the purpose of this research, the detection of bacteria from the genus Salmonella, the total bacteria count, and contamination with Enterobacteriaceae on carcasses originating from Aubrac cattle were pursued.
Sampling from carcasses is carried out according to the SR ISO 17604/2009 standard, utilizing both destructive and non-destructive methods (Figure 1).
In this research, the sponge swabbing method was employed, where a surface area of 100 cm^2 was wiped using the template using sponges soaked in sterile peptone diluent. The wiping procedure was repeated 10 times vertically and 10 times horizontally at the selected site.

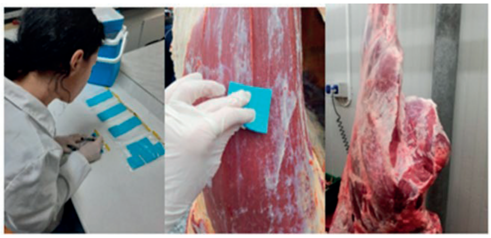

Figure 1. Sampling from carcasses
(original foto)

The sampling was conducted randomly from five carcasses, selecting four areas from each carcass with the highest contamination frequency, resulting in a total surface area of 400 mm^2.After sampling, all samples were packaged and labelled, ensuring they were sealed. The next step involved sending the samples to a specialized laboratory under appropriate conditions to avoid any alterations to their quality at the time of collection.
After sampling, to detect bacteria from the Salmonella genus, the horizontal method according to standard SR EN ISO 6579-1:2017/A1:2020 was used. For conducting the analysis, the following materials and glassware were used: sterile Petri dishes of various sizes, graduated or automated pipettes, sterile Pasteur pipettes or sterile pipettes, a pH meter, test tubes or bottles, a water bath, and apparatus for dry sterilization (oven) or wet sterilization (autoclave).
For preparing culture media and reagents, the following components were used: non-selective pre-enrichment medium: buffered peptone water (BPW); selective enrichment media: Rappaport-Vassiliadis soy broth (RVS broth) and Muller-Kauffman-tetrathionate-novobiocin broth (MKTTn broth); selective solid isolation media: xylose lysine deoxycholate agar (XLD agar); and a second medium of choice.

To make sure the biochemistry was correct, different types of agar (Christensen), physiological saline solution, medium for lysine decarboxylation, and agglutination serums for somatic "O" and "H" antigens were used.
Initially, a 25 g sample was taken for analysis and inoculated with 225 ml of buffered peptone water. The sample was then incubated at a temperature of 37°C ± 1°C for 18 hours ± 2 hours.
In the selective enrichment stage, 0.1 ml of the previously obtained culture was transferred to a test tube containing 10 ml of RVS broth. Additionally, 1 ml of culture was transferred to a test tube containing 10 ml of MKTTn broth. The RVS broth was incubated for 24 hours ± 3 hours at a temperature of 41.5°C ± 1°C, while the MKTTn broth was incubated for the same period of time but at a temperature of 37°C ± 1°C.
After the selective enrichment stage, the first selective medium (XLD agar) was inoculated using a loop with the culture obtained from the RVS broth. Similarly, the second selective medium was inoculated using the culture from the MKTTn broth. The two media were incubated for 24 hours at a temperature of 37°C ± 1°C, and the results were to be examined.
For confirmation, at least one suspicious colony was selected from each plate, and if the first colony was negative, another four colonies were taken. These colonies were streaked on nutrient agar plates and incubated at 37°C ± 1°C for 24 hours (Figure 2).

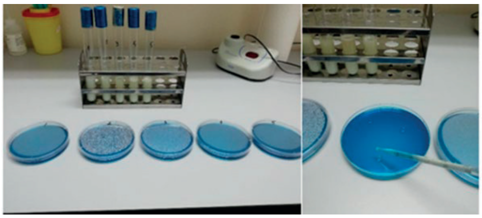

Figure 2. Plate streaking
(original photo)

For determining the total germ count, the horizontal method for enumerating microorganisms at 30°C was used, in accordance with standard SR EN ISO 4833-1/2014. This method involves the use of the following materials and equipment: dry or wet sterilization apparatus, incubator, Petri dishes, pipettes, water bath, colony counting device, and test tubes. Ten milliliters of the sample to be analyzed were mixed with ninety milliliters of physiological saline, which is the horizontal method for counting microorganisms. For inoculation and incubation, one plate was used for each dilution, transferring 1 milliliter of sample using new sterile pipettes for each plate. Subsequently, approximately 12-15 milliliters of PCA culture medium at a temperature of 44-47°C were poured into each Petri dish. The time between preparing the initial suspension or dilutions and pouring the medium into plates did not exceed 45 minutes. The inoculum was carefully mixed with the medium by rotating the Petri dishes, and then the mixture was allowed to solidify with the plates placed on a cool horizontal surface.
After complete solidification of the mixture, the plates were inverted and incubated at a temperature of 30°C ± 1°C for 72 ± 3 hours. At the end of the incubation period, the colonies on the plates were counted using a special device equipped with magnifying lenses, which facilitates colony counting (Figure 3).

Figure 3. Colony Counting
(original photo)

The horizontal method for detecting and enumerating Enterobacteriaceae was determined using standard SR ISO 21528-2/2017. This utilizes the following culture media: buffered peptone water, VRBG agar, glucose agar, brilliant green bile glucose broth, and oxidase reagent. For conducting the test, sterile Petri dishes, sterile test tubes, graduated pipettes, Pasteur pipettes, inoculating loops, and a test tube rack were required.
Non-selective pre-enrichment was carried out by adding 10 ml of buffered peptone water to the sanitation buffer with which the sample was collected. The mixture was homogenized and left to incubate for 18 hours ± 2 hours at 37°C.
For selective enrichment, 1 ml of the obtained culture was transferred to a test tube containing 10 ml of brilliant green bile glucose broth and

incubated at 37°C for 24 hours ± 2 hours. For colony isolation, an inoculating loop was used to streak the surface of VRBG agar, which had been previously poured into a Petri dish (Figure 4). The plate was then incubated at 37°C for approximately 24 hours.

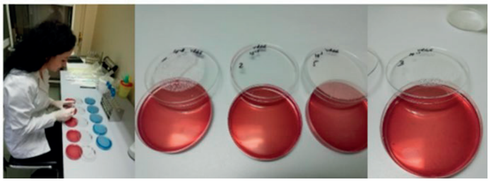

Figure 4. Isolation of Colonies
(original photo)

After the results were read, the number of colony-forming units per square centimeter (CFU/cm²) was calculated using the formula: CFU/cm² = N * F * A * D, where N represents the number of colony-forming units per milliliter of dilution liquid, F is the quantity of dilution liquid in the test tube, A is the investigated surface area, and D is the reciprocal of the dilution factor used.

For confirmation, isolated colonies underwent tests for glucose fermentation and oxidase. Thus, colonies showing positive glucose fermentation and a negative oxidase test were identified as Enterobacteriaceae.

After conducting all analyses, the results obtained were read, interpreted, and subsequently centralized.

RESULTS AND DISCUSSIONS

Results regarding Salmonella contamination

After sample collection, the analysis method described in standard SR EN ISO 6579-1:2017/A1:2020 was used to determine *Salmonella* contamination. From the data presented in Table 1, it is observed that the presence of bacteria from the *Salmonella* genus is not detected in the analyzed samples. Both in standards and in specialized literature, it is specified that *Salmonella* must be absent in the entire sample mass under analysis. Thus, we can conclude that the collected samples originate from healthy carcasses.

Table 1. Results regarding the contamination of bovine carcasses with *Salmonella*

Carcass identification number	Gender		Analysis method
	Male	Female	
1	Absent		SR EN ISO 6579-1:2017/ A1:2020
2	Absent		
3	Absent		
4	Absent		
5	Absent		

Results regarding the total bacterial count

Samples were collected from various areas of bovine carcasses, following standard SR EN ISO 4833-1/2014, and the obtained results are structured in Table 2. For the determination of the total germ count, dilutions of up to 10^2 were used for better interpretation of the results. Subsequently, the read values were multiplied by 10^2. From the obtained result (Table 2), it can be observed that the highest microbial load is found in males (8.2×10^2 CFU/cm^2) on carcasses No. 2 and 5. At the same time, the lowest microbial load (6.0×10^2 CFU/cm^2) was recorded on carcass No. 4 from females. Thus, the values for the total germ count range between 6.0×10^2 CFU/cm^2 and 8.2×10^2 CFU/cm^2, and based on the results, we can appreciate that these values are below the standard's permissible limit, resulting in healthy meat.

Table 2. Results regarding the total bacterial count

Carcass identification number	Gender		Analysis method
	Male	Female	
1	6.4×10^2 CFU/cm^2	8.0×10^2 CFU/cm^2	SR EN ISO 4833-1/2014
2	8.2×10^2 CFU/cm^2	6.1×10^2 CFU/cm^2	
3	7.3×10^2 CFU/cm^2	7.4×10^2 CFU/cm^2	
4	6.4×10^2 CFU/cm^2	6.0×10^2 CFU/cm^2	
5	8.2×10^2 CFU/cm^2	6.2×10^2 CFU/cm^2	
Daily logarithmic mean	**2.86 log CFU/cm^2**	**2.82 log CFU/cm^2**	

Results regarding contamination with Enterobacteriaceae

Similar to the previous examination, carcasses from both males and females were analyzed (SR EN ISO 21528-2/2007). From the obtained results, it can be observed in Table 3 that the presence of bacteria from the Enterobacteriaceae genus was not detected in the analyzed samples. If no presence of Enterobacteriaceae is detected in a microbiological test of beef, this indicates that organisms from the Enterobacteriaceae family were not identified in the tested sample. This result can be considered favorable in terms of food safety, as the absence of Enterobacteriaceae suggests a low or nonexistent level of bacterial contamination in the tested beef.

Table 3. Results regarding the contamination of carcasses with Enterobacteriaceae

Carcass identification number	Gender		Analysis method
	Male	Female	
1	Absent		SR EN ISO 21528-2/2007
2	Absent		
3	Absent		
4	Absent		
5	Absent		

The absence of Enterobacteriaceae is generally an indicator that hygiene and quality control processes have been effective in preventing bacterial contamination.

CONCLUSIONS

In conclusion, the results of the microbiological analyses indicate a high level of hygiene and food safety in the production and handling processes of beef. The absence of *Salmonella*, total bacterial count, and Enterobacteriaceae bacteria in the analyzed samples demonstrates that hygiene standards have been strictly adhered to at all stages of the process, from slaughter to packaging. These positive results are essential to ensuring consumers that the final product is safe and suitable for consumption. Furthermore, these findings underscore the effectiveness of hygiene control measures and good manufacturing practices applied in the beef industry. As food safety is a major concern for consumers, these results validate the food industry's commitment to providing superior-quality and safe products for consumption.

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SMART TECHNOLOGIES AT LIVESTOCK FARMS

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Abstract

At the livestock farm level, smart technologies are used mainly for identifying and locating animals, monitoring them to assess welfare and body condition, assessing and predicting performance, health and reproductive status. Data obtained using various measuring devices (sensors), most often appropriately transformed and integrated, enable early detection of physiological events that routinely occur in the animal's life (estrus, upcoming parturition), as well as undesirable events, such as metabolic disorders and diseases (mainly of the udder and limbs). Also, constant monitoring of animals, resulting in the collection of large sets of data supports the genomic evaluation of the breeding values. Thanks to smart technologies, it is possible to assess the correctness of nutrition, the quality of pastures and the impact of animals on the environment, including the amount of greenhouse gas emissions, as well as to control the microclimate of livestock buildings and to predict the economic efficiency of production. Moreover, these technologies are used for ensuring the safety and quality of animal products in the chain from the producer to the consumer.

Key words*: data sets, livestock, management, monitoring, smart technologies.*

INTRODUCTION

The rapidly growing world population creates the need to constantly increase the supply of food products, especially in developing countries. Expert estimates indicate that global demand for animal products such as meat, milk and eggs will increase by more than 70% in the next three decades (Godfray et al., 2010). This results in the taking over of more and more areas for agricultural use and a significant intensification of agricultural production in many countries. The intensification of plant and animal production by increasing specialization, scale of activities and unit inputs, enables achieving high yields and usually improving economic efficiency. Unfortunately, excessive intensification of livestock production may lead to deterioration of animal welfare, health and longevity, contamination of soil and water with artificial fertilizers used in the cultivation of feed plants and large amounts of animal excrement introduced into the environment, increased emissions of greenhouse gases (mainly by ruminants), reduced biodiversity, lower quality of food products and, indirectly, also deteriorated people's health. Issues related to production conditions, product quality and the impact of farms on the environment are also the subject of concern in increasingly aware societies. Therefore, contemporary agriculture, including livestock production, faces complex economic, environmental and social challenges. It must reconcile the increase in the production of high-quality food and ensuring adequate profitability while limiting the unfavourable effects of the taken actions. The recommended solution is economically, environmentally and socially sustainable intensification of agriculture (Garnett et al., 2013; Ponisio & Kremen, 2016; Lovarelli et al., 2020).

Obtaining satisfactory results, both in intensive and more sustainable livestock production, requires skilful management of herds maintained in various natural, technical and market conditions. The use of scientific research results and advanced engineering and technical solutions has actually contributed to the enormous progress observed in livestock production over the last several decades. Proper herd management requires an individualized approach to animals, possible thanks to innovative engineering techniques and digital technologies, allowing for full automation of animal monitoring as well as the collection and processing of large production, physiological, behavioural and environmental data sets. Moreover, generating and sending electronically information supporting optimal decision-making as well as using automats and robots in many farm activities is increasingly common.

MATERIALS AND METHODS

The combined use of sensor technology, the related algorithms, interfaces, and applications in livestock husbandry is called Precision Livestock Farming (PLF) (Kleen & Guatteo, 2023). According to Tullo et al. (2019) PLF can be defined as "the application of process engineering principles and techniques in livestock breeding for automatic monitoring, modelling and management of livestock production".
PLF consists in optimal, broadly understood, control of the herd and technical devices using real-time, comprehensive, automated monitoring of physiological, behavioural and production indicators for individual animals, indicators describing the farm environment and functioning of machines and devices, as well as collecting the information on economic, environmental and social phenomena that may affect the efficiency of livestock farming. PLF uses the Internet of Things (IoT), information and communication technologies (ICT) and cloud computing for collecting, processing, and transmitting information in electronic form (Benjamin & Yik, 2019).
Sensor technology enables comprehensive monitoring providing a huge amount of information (Big Data) about animals, farm environment, and broadly understood economic, environmental and social aspects of production processes. Big Data are large and complex data sets, requiring special tools and techniques for processing and analysis.
Animal monitoring requires their identification and location. For this purpose, collars with relatively large transponders using high-frequency radio waves (Radio Frequency IDentification - RFID) are commonly used. There are also miniaturized and cheaper devices that use low-frequency radio waves, such as sensors placed on the ear, intraruminal boluses or devices implanted under the skin. Data on animals and farm environment recorded by sensors placed on and inside the body of animals, sensors installed in buildings, machines and farm equipment, delivery vehicles and vehicles transporting animals to the slaughterhouse may be in numerical, visual or acoustic form. Monitoring techniques include the use of activometers, including pedometers and three-axis accelerometers, as well as wireless telemetry, cameras and 2D and 3D scanners, automatic scales with the function of measuring hoof pressure, as well as acoustic chewing sensors. Images and sounds are usually processed using appropriate algorithms to obtain numerical data that can be subjected to further analysis. Additionally, the data on climate and weather conditions, soil quality, crop volume, operational activities of enterprises, market situation and consumer preferences are collected.
The basis for Precise Livestock Farming is the use of artificial intelligence (AI) - a technology that, by simulating intelligent human behaviour, effectively supports the optimization of decisions and the automation of activities and processes in livestock management. According to Russell (2016), artificial intelligence is a set of methods combining data, algorithms and computing power capable of performing tasks normally requiring human intelligence, such as sensory perception, speech recognition, decision-making, and forecasting. AI includes machine learning (ML) and deep learning (DL) algorithms, with special application of artificial neural networks (ANN) (Berckmans, 2014) (Figure 1).

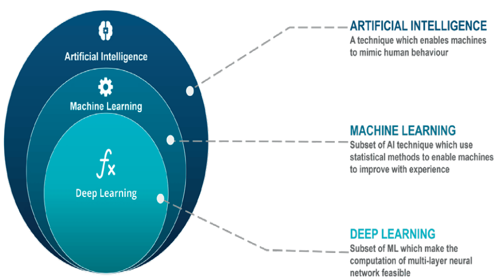

Figure 1. Basic classification of artificial intelligence

Machine learning includes automated analytical systems that learn themselves over time and as more data is acquired. ML tasks are classified into different categories - depending on the type of learning (supervised /unsupervised) and the learning model (classification, regression, clustering and dimensionality reduction). There are also learning models adapted to the implementation of specific tasks (Liakos et al., 2018) By means of self-learning algorithms, computers independently, without the need for programming, analyse data and, taking into account the variability of phenomena and requirements, automatically adjust models in order to acquire new knowledge and increase the ability to solve problems of detection, recognition, classification, prediction or decision choice. Machine learning techniques and algorithms are shown in Figure 2. When ML models encounter new data, they can adapt and use it in the analyses. This technique is closely related to data mining, conducted to obtain information and knowledge as well as search for patterns and relationships. The ability to predict events and identify risk factors is also particularly important (Akbar et al., 2020). The growing interest in ML results from the need to analyse increasingly larger data sets from various sources, including huge Big Data resources and the emergence of much more efficient computing technologies and much cheaper data storage platforms. However, Valletta et al. (2017), conducting an extensive review of the applications of machine learning to assess animal behaviour, concluded that the current data collection capabilities exceed the capabilities of their analysis using classical statistical methods. This applies especially to complex and multidimensional data sets, including traits with non-linear or difficult to determine relationships.

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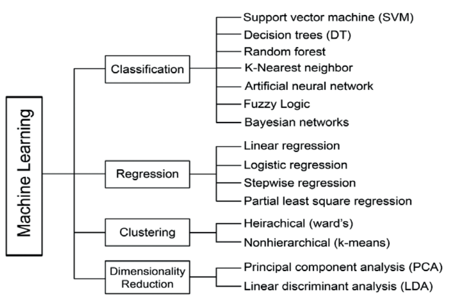

Figure 2. Machine learning techniques and algorithms (Cihan et al., 2017)

A subcategory of machine learning is deep learning (DL), based on artificial neural networks (ANN) (Lu, 2019). The structure of artificial neural networks consists of many layers of input, output and hidden data. When an artificial neuron receives a numerical signal, it processes it and transmits the information to other neurons that are connected to it. Just like in the human brain, strengthening neurons allows for better pattern recognition, knowledge acquisition, and overall learning. Huge amounts of complex and distributed data are used to gradually obtain more and more accurate results.

DL technology has greatly improved computers' ability to detect, classify, and recognize data, generally, the ability to "understand" it. It is widely used in areas that require operations on non-numerical data, e.g. image classify-cation, speech recognition, object detection and describing the content of data sets.

RESULTS AND DISCUSSIONS

Modern livestock management involves an individual approach to animals and taking into account existing production, environmental, economic and social interactions. A great facilitation in this respect is the increasingly widespread access to intelligent engineering and information-communication technologies.

The application potential of technologies using artificial intelligence (PLF with Big Data) was demonstrated by Lokhorst et al. (2019) in a review of 142 papers published between 1994 and 2017. A significant increase in the number of publications was observed around 2010, and the largest share were the papers dealing with the application of PLF in dairy cattle. Most studies described the use of supervised machine learning methods, classification techniques and time series analysis algorithms. Slob et al. (2021), conducting a systematic review of 427 papers on the use of machine learning in dairy farm management, found that 55% concerned the detection of diseases, mainly mastitis, and the remaining two categories were the prediction of production volume (26%) and milk quality (19%). 23 algorithms were identified, among which the best results were obtained using decision trees. Based on 97 papers published between 2015 and 2020, Cockburn (2020) concluded that ML algorithms using integrated data from various sources are a promising decision support tool in physiology, reproduction, behaviour and nutrition of dairy cattle. Despite the wide use of this method, mainly for the prediction, most of the tested algorithms still did not demonstrate satisfactory correctness, which limits their implementation in practice. According to the author an improvement in forecast accuracy can be achieved by increasing the amount of training data, collected over a longer period of time and coming from a larger number of farms.

At the farm level, artificial intelligence is used, among others: for identifying and locating animals, monitoring them in order to assess and predict performance, well-being, condition, health and reproductive status, as well as for determining the level of greenhouse gas emissions or controlling the microclimate of livestock buildings. Data obtained using various measuring devices (sensors), most often appropriately transformed and integrated, enable early detection of physiological phenomena that routinely occur in the animal's life cycle (estrus, upcoming parturition), as well as undesirable phenomena, such as metabolic disorders and diseases (mainly udders and limbs). Also, they can be used for prediction of reproductive performance, production volume and its efficiency, assessment of nutrition correctness, pasture quality and environmental impacts, including greenhouse gas emissions, control of the microclimate of livestock buildings and improvement of working conditions on the farm.

Constant monitoring of animals and forecasting events by learning based on past behaviour, rules and available data is indispensable for supporting optimal decisions resulting in the improvement of performance, welfare and health of individual animals and the entire herds, as well as the state of farm environment, the increase in production and economic efficiency, and reduction of the negative impact of production on the natural environment (Halachmi et al., 2019, Neethirajan, 2020). Early detection of threats and deviations from normal states and possibly indication of their causes enable breeders to take appropriate preventive and adaptive actions. The use of modern technologies for monitoring of animals and environment in the so-called smart farms allow to carry out numerous tasks and processes using automated equipment and even robots. They include individualization of feeding, sorting animals, and controlling the microclimate of livestock buildings, among others (Cihan et al., 2017). Moreover, artificial intelligence is used to ensure the safety and quality of animal products in the chain from producer to consumer (Zhu, 2019).

The main areas of application of intelligent technologies in animal production are presented in Figure 3.

CONCLUSIONS

The use of artificial intelligence, Big Data, the Internet of Things and Internet cloud computing for collecting, processing and transferring data and information on the farm and in the chain from producer to consumer, called "Smart Farming" (Intelligent Agriculture), is no longer just a futuristic concept, but it is part of the technological revolution, currently called Agriculture 4.0 (O'Grady & O'Hare, 2017; Rose & Chilvers, 2018; Wolfert et al., 2017).

Thanks to intelligent digital technologies, it is possible not only to constantly monitor animals, but also to comprehensively analyze production, physiological and behavioral data, which favors a more holistic approach to livestock management on the farm, during transport and slaughter. The modern technologies allow not only to better manage herds in various natural, technical, market and social conditions, but also to collect data used in the genetic improvement of production and functional traits. Summarizing, they significantly contribute to the increase in food production needed to feed the world's population, but also to more sustainable livestock farming owing to optimization of production efficiency in the context of expenditure incurred, used natural resources, appropriate animal welfare, quality of food products and protection of environment (Tedeschi et al., 2021).

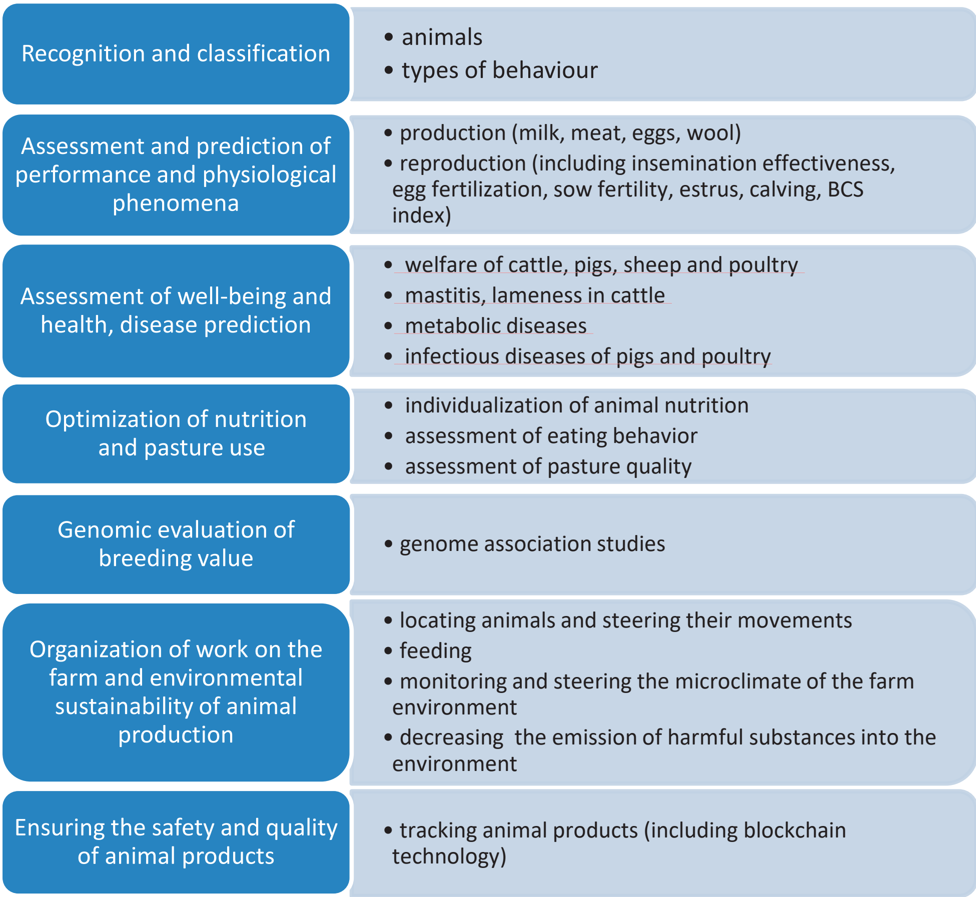

Figure 3. Main applications of artificial intelligence algorithms in livestock farming

An increased use of intelligent technologies in both intensive and more sustainable, and even extensive, livestock production systems will surely be continued but due to the still relatively high, although clearly decreasing costs, in the near future it will probably be limited to relatively large farms (Berckmans, 2014; Norton et al., 2019). An important driver may be the possibility of reducing employment as a result of using the innovative solutions in farm activities (Vaintrub et al., 2020).

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THE EFFECT OF LACTATION STAGE ON THE COMPONENTS OF MILK, DURING THE GRAZING PERIOD, IN BUFFALOES COWS FROM THE ROMANIAN BUFFALO BREED

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Abstract

Currently, approximately 20.000 buffaloes are raised in Romania. The present study was carried out to observe the effect of lactation stage on the components of raw milk obtained from buffaloes of the Romanian Bubaline breed. 120 milk samples were collected during the morning and evening milking, during the grazing period, from 60 buffaloes in different stages and ranks of lactation. From the collected samples, the content of fat, protein, lactose, fat-free dry matter (NFD), and total dry matter (TU) were determined. The individual analysis of milk samples from buffaloes revealed significant differences in terms of the variation of these parameters, the research carried out revealed differences determined by the stage of lactation in the same reference season. Therefore, the results of the present research indicated that the stage of lactation, during the grazing season, significantly influences the chemical parameters of milk, regardless of the lactation rank.

Key words: *buffaloes, fodder, milk, lactation, pasture.*

INTRODUCTION

In Romania, raising buffaloes is a traditional activity, they are raised and exploited for milk-meat, but also for their traction force. During the communist regime, the herd of buffaloes reached the historical peak of 228,000 heads, and in 2004, Romania ranked second in Europe in terms of buffalo growth with 100,000 heads. During the last decades, the number of buffalo herds has decreased significantly, reaching in 2022 17,818 heads with a production of 15,526 tons of milk (INS, 2023).

According to the Food and Agriculture Organization (FAO), the global production of buffalo milk in 2022 was 143 million tons, representing about 19% of the total milk production obtained from buffalo cow (FAO, 2024). The attraction and investment in buffalo milk has been steadily increasing every year due to its unique taste and nutritional value, including much lower cholesterol, being used in a wide range of commercial products. Buffalo milk is of a special quality and contains 40% less cholesterol than cow's milk, being preferable for patients with atherosclerosis, dyslipidemia and cardiovascular diseases.

The main composition of buffalo milk is defined by the content of fat, protein, lactose, non-fat dry matter (NF) and total dry matter (TS). The distinguishing feature of buffalo milk is its high fat and calorie content, along with its dry matter. Changes in milk composition are influenced by several factors such as genetics, milking time, type of diet, age, udder hygiene and season.

In this context, the aim of the research is to examine the effects of the season and stage of lactation on the chemical composition of milk obtained from female buffaloes regardless of the rank of lactation of the Romanian Buffalo breed. The research results can be practically applied to improve the management of buffalo farming and the quality of the milk produced, thus contributing to the revitalization of this important agricultural tradition. Besides the economic benefits, raising buffaloes also has ecological advantages, helping to maintain biodiversity and traditional landscapes. Moreover, modern technologies and innovative practices in buffalo raising play a crucial role in increasing productivity and animal health. Buffalo milk, recognized for its nutritional benefits, rich in essential vitamins and minerals, is increasingly appreciated for its contribution to human health.

Support programs and public policies, such as SCZ in buffaloes cows, ANTZ (Transitional National Aid for Bovines), the state aid scheme for animal breeding and performance control, and the Agri-Environment and Climate Measure for Raising Animals from Local Breeds in Danger of Abandonment - the Romanian Buffalo, encourage farmers to maintain this tradition. These programs reflect the cultural and traditional importance of raising buffaloes in Romania and support farmers in their efforts to preserve and revitalize this essential agricultural sector.

MATERIALS AND METHODS

The study was carried out on a sample of 60 buffaloes from the Romanian Buffalo breed, in different stages of lactation. The Romanian buffalo is characterized by morphological type of milk, waist 132 cm, body weight 490 kg, age of first calving 43.92 months, duration of lactation 474.74 days, average amount of milk per lactation 1669.03 kg, average amount of milk at first lactation 1385.55 kg, fat 7.65% and protein 4.30%. They have a well-developed stature, large head and horns, thin neck, long and broad back, wide rump and short legs. The animals were grazed between July and September and were fed green mass at discretion and concentrated fodder, in the amount of 2 kg/day/head. The milking process was done mechanically, using a mechanical drum milking plant.

In the study, two milk samples were collected from each buffalo, at morning and evening milking, resulting in a total of 120 milk samples. Buffaloes were divided into three distinct groups based on lactation stage: early lactation (1-2 months), mid-lactation (3-6 months) and late lactation (7-9 months).

Milk samples (250 ml/sample) were collected under aseptic conditions in sterile bottles and then transferred to the laboratory for detailed analysis. The analysed parameters included the content of fat, protein, lactose, dry matter without fat (SNF) and dry matter (SU).

The determination of the content of fat, protein, lactose, dry matter without fat (SNF) was carried out with the help of an ultrasonic milk analyser "EKOMILK-ULTRA". The data obtained were subjected to a statistical analysis to evaluate the average differences between the results of the determinations, expressed according to the standard deviation, using the ANOVA analysis of variance, with a level of significance set at $P>0.05$.

In addition, each milk sample collection procedure followed strict sanitation and handling protocols to ensure the integrity and validity of the data obtained.

These methods were applied to ensure accurate and relevant results were obtained in the study carried out on Romanian buffaloes.

Figure 1. Values obtained from a milk sample (own souce)

RESULTS AND DISCUSSIONS

The results of the physical analysis performed on the milk samples taken during the grazing period are presented in Table 1.

Table 1. Results of chemical analysis of buffalo milk according to stage of lactation

Parameter/Stage of Lactation (N)	Fat (%)	Protein (%)	Lactose (%)	SNF (%)	SU (%)
The beginning of lactation N = 20	7.11± 0.21	4.59± 0.26	4.51± 0.22	9.36 ± 0.14	17.19 ±0.07
Mid lactation N = 20	7.42± 0.25	4.46± 0.24	4.39± 0.27	8.75 ±0.14	17.36 ±0.11
End of lactation N = 20	7.53± 0.23	4.28± 0.16	4.25± 0.33	8.96 ± 0.12	17.62 ±0.21

(%) = (g/100 ml); n = number of determinations

Fat

During the grazing period, the fat content of buffalo milk can be influenced by the quality and type of forage available. Grazing on fresh, nutrient-rich grass can contribute to higher milk fat content compared to feeding nutrient-poor forage. One of the most important components of buffalo milk is fat. The fat content not only directly affects the nutritional and economic value of milk, but also has an effect on the organoleptic properties. The fat content of raw milk is so important that milk processors tend to set the price based on the fat content. The quality of dairy products such as cheese, cream and butter depends largely on the quantity and quality of milk fat. In particular, buffalo mozzarella, famous for its soft and elastic texture, owes its superior qualities to the fat of buffalo milk. The fat in buffalo milk is a valuable source of fat-soluble vitamins such as vitamins A, D, E and K, which are essential for healthy bones, skin and the immune system. Regular consumption of buffalo milk can help improve your overall health due to its nutrient-rich profile and beneficial fatty acids. The amount of milk fat during the grazing period recorded an average of 7.35%. The highest amount of fat was recorded at the end of lactation, 7.52%, and the lowest at the beginning of lactation, 7.11%, the differences being statistically significant. Compared to cow milk, buffalo milk has a significantly higher fat content, averaging 7.42% in mid-lactation and reaching up to 7.53% in late lactation during the grazing period. In comparison, cow's milk generally has a lower fat content, around 3.5%. Correct management of the fat content of buffalo milk is essential to ensure high quality dairy products appreciated by both consumers and processors.

Protein

Buffalo milk protein is a component that influences the processing of milk into cheeses and attracts attention due to its role in the final quality of dairy products. Proteins influence coagulation time and syneresis, essential factors in the cheese-making process (Bota A., 2023). Casein, the main milk protein, accounts for about 80% of the total protein content and is responsible for the texture and elasticity of cheeses. Protein content varies according to the lactation period, with significant values recorded at different stages of this cycle. During the grazing period, the average protein content is about 4.44%. The highest protein concentration, 4.58%, is observed in early lactation, reflecting a period of maximum milk production and nutrients. In contrast, towards the end of lactation, the protein content decreases slightly to 4.28%, but significant statistical differences underline the importance of this variation in the context of milk production and optimal use of buffalo resources. These fluctuations in protein content demonstrate the complexity and dynamics of the lactation process in buffaloes, directly influencing the quality and yield of the final dairy products.

Buffalo milk has an average protein content of 4.46% in mid-lactation and peaks at 4.59% in early lactation during the grazing period, while cow milk has an average protein content of about 3.2-3.4%.

The table below provides a comparative picture of the composition of buffalo milk from different countries and breeds as reported in various studies.

Table 2. The chemical composition of buffalo in different countries (%) (according to M.H. Adh El-Salam, S. El Shibiny, 2011)

Fat	SU	SNF	Protein	Lactose	Country/ Author
7.0± 1.3	16.6± 2.6	-	3.73± 0.82	4.57± 0.23	Argentine/ (Patino s.a., 2003) *Murrah
7.6± 1.8	16.8± 2.6	-	3.73± 0.88	4.51± 0.21	Argentine/ (Patino s.a., 2003) *Mediterranean MurrahxR
8.8± 0.3	18.4± 0.2	-	5.20± 0.14	4.55± 0.01	Argentine/ (Patino & Stefani, 2005)
8.4± 9.0	-	-	4.24± 4.45	4.90± 5.05	Azerbaijan/ (Akhundov & Farzalieva, 1979)
8.4± 0.3	17.7± 0.4	9.5± 0.2	3.97± 0.06	4.80± 0.1	Bangladesh (Khan s.a., 2007) *Swamp buffalo
7.34 ±0.5	16.7± 0.1	9.2± 0.2	3.77± 0.26	4.76± 0.18	Bangladesh (Khan s.a., 2007) *River buffaloes

Argentina (Patino & Stefani, 2005) - The study on Murrah buffaloes from Argentina reported a protein content of 5.20%, the highest value seen in the table. This high value can be attributed to efficient management practices and optimal feeding conditions, which contribute to high quality milk production.

Azerbaijan (Akhundov & Farzalieva, 1979) - The Azerbaijan study reported variable protein values, with a content of 4.24% and 4.45%. These differences may reflect variability in the breeds studied and in environmental and feeding conditions.

Bangladesh (Khan et al., 2007) - The Bangladesh study observed a protein content of 3.97% for Swamp buffaloes and 3.77% for river buffaloes. These values are lower compared to those in other studies, indicating possible differences in diet and animal management.

Lactose

There is only one carbohydrate in milk, lactose, which influences its sensory properties. This disaccharide, composed of glucose and galactose, gives milk a slightly sweet taste and contributes to its distinctive flavor. Lactose comes in three forms: hydrated alpha, anhydrous beta and an amorphous form, which consists of a mixture of alpha and beta lactose in a ratio of 1:5 (Coroian, 2009). Lactose partially decomposes at temperatures of 70-80ºC with the formation of organic acids in particular, formic and lactic acid. Lactose plays an important role in the fermentation processes needed to make yogurt and other fermented products. Lactic acid bacteria use lactose as an energy source, producing lactic acid and thus contributing to the texture and taste of the final product. The results of the determinations showed that there is a significant difference ($p \leq 0.05$) in terms of lactose content between the milk obtained at the beginning of lactation (4.50%) and that at the end of lactation (4.24%), this being in protein balance. The average lactose in milk obtained from buffaloes at different stages of lactation is 4.04%.

Buffalo milk has an average lactose content of 4.39%, reaching a maximum of 4.51% in early lactation during the grazing period, while cow milk has an average lactose content of about 4.8-5%.

Lactose has numerous health benefits, including providing a quick source of energy and aiding the absorption of calcium and other minerals. Lactose intolerance is caused by a deficiency of the enzyme lactase, which is needed to digest lactose. Buffalo milk, although high in lactose, can be processed to produce lactose-free dairy products so that it is also accessible to those with intolerances.

Pece et al. (2009) stated that the lactose content of the Romanian Buffalo breed milk was 4.8%, for all seasons.

To better understand the variability in the composition of buffalo milk in different regions of the world, data from various studies are presented below. This information illustrates the differences in lactose, fat and protein content by country and the conditions specific to each study.

Table 3. The chemical composition of buffalo milk in different countries (%) (according to M.H. Adb El-Salam, S. El Shibiny, 2011)

Fat	SU	SNF	Protein	Lactose	Country/Author
8.1± 1.9	-	-	4.65± 0.48	-	Italy (Tufarelli s.a. 2008)
7.6± 0.1	-	9.8± 0.1	4.11± 0.02	-	Pakistan (Imran s.a.2008)
7.0± 0.6	-	-	4.35± 0.02	5.21± 0.11	Pakistan (Arian s.a., 2008)
7.64	18.16	-	4.69	4.85	Romania (Velea C s.a., 2006)
7.8	17.5	-	4.0	4.9	Romania (Vidu Livia, 2007)
7.1± 1.4	16.6± 1.6	9.6± 0.8	4.40± 0.51	-	Turkey (Sekerden s.a., 1999)

Italy (Tufarelli et al., 2008): Buffalo milk has a high content of fat (8.1±1.9%) and protein (4.65±0.48%), reflecting the superior nutritional qualities of buffalo milk.

Pakistan (Imran et al., 2008): Buffalo milk has a protein content of 4.11±0.02% and a SNF (fat free solids) level of 9.8±0.1%, indicating a balanced nutritional profile.

Pakistan (Arian et al., 2008): Lactose content reaches 5.21±0.11%, a relatively high level, suggesting a more pronounced sweetness.

Romania (Velea et al., 2006): Lactose content is 4.85%, indicating slight seasonal and regional variability in Romanian buffalo milk.

Romania (Vidu, 2007): In this study, the lactose content was 4.9%, reinforcing the idea of a consistent lactose profile in Romanian buffalo milk.

Turkey (Sekerden et al., 1999): This study revealed a protein content of 4.40±0.51%, with a variability in SNF content of 9.6±0.8%, indicating the influence of local conditions and feed on composition of buffalo milk.

Solid non-fat

One of the important parameters when evaluating the quality of milk is the content of solid non-fat (SNF). The SNF in buffalo milk includes protein, lactose, vitamins, calcium, and trace minerals. These components significantly contribute to the nutritional value of the milk, influencing both its physico-chemical and organoleptic properties. A high SNF content contributes to the density and texture of dairy products such as yogurt, cheese, and milk powder. It has been found that the lactation stage affects the fat-free solids, with a tendency to decrease from early to late lactation (9.36-8.95%). Standardizing the ratio of fat to non-fat solids in raw milk is essential for dairy production.

The study revealed a significant difference in the dry matter content in different stages of lactation, with an increasing trend from the beginning of lactation towards its end (17.36±0.11% in the peak period of lactation). Buffalo milk has an average SNF content of 8.75% in mid-lactation, reaching a maximum of 9.36% in early lactation during the grazing period, while cow milk has an average SNF content of about 8.5-9%.

CONCLUSIONS

The results of the present study indicate that lactation stage affects certain components of milk obtained from buffaloes during the grazing season. Milk obtained from buffaloes with a stage of lactation towards the end, recorded values of the fat content of 7.53±0.23%, significantly higher compared to milk obtained from buffaloes in the beginning and middle stages of lactation (7.11±0.21%, respectively 7.42±0.25%). On the other hand, the protein and lactose content of milk had significantly higher values in buffaloes with early lactation stage (4.59±0.26% protein and 4.51±0.22% lactose), compared to those in the end stage of lactation (4.28 ±0.16% protein and 4.25±0.33% lactose). Regarding the percentage of dry matter in milk, significantly higher values are noted in buffaloes in the stage of advanced lactation, compared to those with early and middle lactation. The stage of buffaloes, being a physiological process, cannot be changed, instead good management practices such as proper feeding and maintenance during the grazing period could be the physico-chemical properties of the milk obtained. Adjusting feed composition and quality to meet the nutritional needs at different lactation stages could further enhance milk quality.

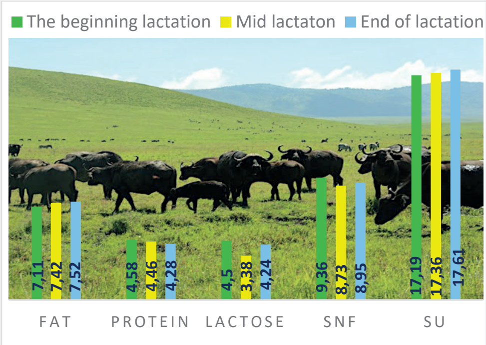

Figure 2. Composition of buffalo milk at different stages of lactation during the grazing period

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IMPACT OF MANGANESE HYDROXYCHLORIDE ON EGG QUALITY, ANTIOXIDANT CAPACITY, BONE CHARACTERISTICS, AND MINERAL EXCRETION IN LAYING QUAIL

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Abstract

This research investigated the impact of different concentrations of manganese hydroxychloride (MnH) on productive performance, egg quality, antioxidant status, tibia characteristics, and mineral excretion in laying quails. A total of 125 female ten-week-old female quails, were divided into five groups with five subgroups, each containing five quails. The birds were fed isoenergetic and isonitrogenous diets with different levels of MnH (containing 55 g/100 g Mn) at 18.86 mg/kg (basal diet), 40, 60, 80, and 100 mg/kg for twelve weeks. Results indicated that MnH supplementation enhanced egg production and feed intake (P < 0.05) compared to the non-supplemented group. The optimal eggshell quality, including shell-breaking strength, thickness, and weight, was observed at 80 mg/kg MnH (P<0.01) compared to the basal diet. Regarding the antioxidant capacity in the yolk, the yolk's 2,2- diphenyl- 1- picrylhydrazyl (DPPH) value increased significantly (P<0.01) with 100 mg/kg MnH supplementation, while malondialdehyde (MDA) values remained unaffected across all groups. Increasing dietary MnH levels elevated Mn excretion in faeces and reduced copper levels (P<0.01). However, MnH addition adversely affected tibia biomechanical properties compared to the basal diet (P<0.01), with no significant impact on tibia mineral accumulation (P>0.05). These results suggest that including 80 mg/kg MnH in laying quail diets would be adequate to improve certain aspects of production and eggshell quality, although its impact on bone parameters requires further investigation.

Key words: *antioxidant, egg, manganese hydroxychloride, minerals, quail.*

INTRODUCTION

Manganese (Mn) is a crucial trace mineral for avian nutrition, vital for ensuring animal health, egg quality, and performance development (Jasek et al., 2019). This importance stems from Mn's significant role in enzymatic systems responsible for the metabolism of lipids and carbohydrates. For instance, Mn activates glycosyltransferase, an enzyme essential for producing glycosaminoglycans and uronic acid necessary for bone and eggshell growth (Xiao et al., 2014; Khoshbin et al., 2023). Additionally, Mn is a metalloenzymes component that controls mitochondrial oxidative stress by reducing superoxide to peroxide, which is then converted into water (Bozkurt et al., 2015; Noetzold et al., 2020). Thus, a diet deficient in Mn can compromise poultry health, increase production costs, and negatively impact the environment (Gül et al., 2023).

To meet Mn intake needs, both inorganic and organic Mn sources are commonly used in avian feed, alone or in combination (Jasek et al., 2019). Organic Mn sources generally offer higher bioavailability and stability than inorganic sources like sulphates and oxides (Olgun, 2017; Jasek et al., 2019). Recently, the avian industry has shifted towards using novel mineral additives to enhance performance, improve egg quality, reduce inclusion rates, and minimise environmental damage (Jasek et al., 2020; Jiang et al., 2021; Groff-Urayama et al., 2023; Olgun et al., 2024). In this context, hydroxychloride forms of Mn present a promising alternative as an inorganic source. The advantages of using hydroxychloride trace elements in animal nutrition are their unique crystalline structure, characterised by stronger covalent bonds between hydroxyl groups and chloride ions, unlike ionic forms that contain carbon ligands. This structure results in lower solubility in neutral or water solutions and higher solubility in acidic environments, such as the upper intestine. Consequently, hydroxychloride forms exhibit reduced reactivity with other dietary components compared to ionic sources, leading to delayed release during digestion, enhanced absorption, and decreased environmental excretion (Huang et al., 2013; Jasek et al., 2019; Groff-Urayama et al., 2023; Olgun et al., 2024).

The global population of Japanese quails is increasing, driven by their ease of management, high demand for eggs and meat, rapid production cycles, and economic sustainability (Sarmiento-García et al., 2023). Despite these advantages, there has been relatively little research on their nutritional requirements; instead, information has mostly been based on data published 30 years ago by the National Research Council (1994). While numerous studies have addressed macronutrient needs such as energy, protein, calcium, and phosphorus, there is a notable lack of research on the Mn requirements of quail.

The National Research Council (1994) recommended a Mn requirement of 60 mg/kg for laying quails. In contrast, Gökmen & Bahtiyarca (2018) suggested that certain developmental and egg quality parameters could be enhanced with diets containing 81.56 mg/kg of Mn (including 21.56 mg/kg dietary content plus 60 mg/kg supplementation). However, to our knowledge, this is the only study available on the dietary administration of Mn (whether inorganic or organic) to laying quails, and there is no literature on the supplementation of Mn hydroxychloride (MnH) in quail diets. This research aims to address this gap by examining the effects of increasing levels of MnH supplementation on the performance, egg quality, antioxidant capacity in eggs, mineral excretion, and bone properties of quails. The study seeks to determine the optimal MnH dosage level to support these parameters effectively.

MATERIALS AND METHODS

There are no particular restrictions for keeping experimental animals because the following investigation has been conducted with farm animals. Nevertheless, the standards outlined in the European Animal Protection Policy (EPCEU, 2010) as well as the principles reported in the 1964 Declaration of Helsinki were met throughout the experimental study.

One hundred twenty-five female quails (*Coturnix coturnix Japonica*) weighing 255.50 ± 16.27 g and ten weeks old have been chosen for this investigation. A local indoor farm in Selçuklu, Konya, Türkiye (38°1′36″, 32°30′45″) employed a fully randomised design for 84 days. There are five treatment trial groups, each comprising five subgroups and five quails. The quails have been aleatorily assigned into five identical battery cages (30 x 45 cm). Each cage has been sterilized, well-ventilated, and cleaned. Each pen had a temperature of 22°C (± 2.0) and a 16-hour lighting regime. For *ad libitum* access to food and water, individual feeders and drinks have been placed in each pen.

The basal diet, consisting of corn and soybean meal, was provided to all quails for 84 days. This diet contains a 200 g/kg crude protein (CP) content and 18.86 mg/kg of Mn, which translates to 2900 kcal of metabolizable energy per kilogram of feed (Table 1). Initially, the basal diet included 0 mg/kg of supplemental MnH with 18.86 mg/kg of inherent Mn. MnH (containing 55 g/100 g of Mn) was subsequently added to the basal diet at doses of 40, 60, 80, and 100 mg/kg to create the experimental diets. The foundational diet, offered in a mash form, was formulated to fulfil the nutritional needs of laying quails as outlined by the National Research Council (1994), except for Mn. The chemical composition of this basic diet was assessed using the methods specified by the AOAC (2006), and the details are presented in Table 1.

Table 1. Basal diet and its nutrient composition

Ingredients	g/kg	Nutrients	g/kg
Corn	544.00	ME (kcal/kg)	2899.08
Soybean meal (460 g/kg CP)	344.00	Crude protein	200.13
Soybean oil	36.50	Crude fibre	28.30
Limestone	56.00	Crude fat	58.38
Dicalcium phosphate	11.40	Moisture	128.32
Salt	3.50	Lysine	10.90
Premix[1]	2.50	Methionine	4.49
DL-methionine	2.10	Cystine	3.73
Total	1000.00	Calcium	24.98
		Total phosphorus	6.37
		Available phosphorus	3.49
		Manganese (mg/kg)	18.86

[1]Premix provides per kg feed: vitamin A, 8000 IU; vitamin D_3, 3000 IU; vitamin E, 5 mg; vitamin K, 2 mg; folic acid, 1 mg; biotin, 0.1 mg; niacin, 50 mg; pantothenic acid, 15 mg; vitamin B12, 0.02 mg; pyridoxine, 4 mg; thiamin, 3 mg; riboflavin, 10 mg; iodine, 1.0 mg; copper, 10 mg; iron, 50 mg; selenium, 0.42 mg; zinc, 60 mg.

Quails were randomly assigned into various experimental groups to evaluate their performance indicators based on methods described by Olgun et al. (2022) and Sarmiento et al. (2023). The assessment focused on various parameters such as body weight gain, feed intake, feed conversion ratio (FCR), and egg production. Daily feed consumption was measured by the feed given and subtracting the leftover feed in the feeders. Body weight gain was tracked by weighing the quails at the beginning and end of the study period. The feed conversion ratio was computed as the ratio of feed consumed to body weight gained. Egg production was monitored by counting the number of eggs laid by each group throughout the experiment. The egg-laying rate was calculated as the daily number of eggs produced, expressed as a percentage of the total number of quails in each group.

The egg laboratory at Selcuk University, Konya, Türkiye's Faculty of Agriculture conducted the following analyses. All eggs collected over the last three days of the trial have been evaluated for both internal and exterior quality requirements at room temperature. Broken, damaged, and cracked eggs have been counted throughout the experiment and expressed as a percentage of the total number of eggs (n=300). All measurements of eggshell quality have been done using the techniques described by Olgun et al. (2022).

Lipid peroxidation of the yolk in triplicate on 100 fresh eggs was assessed using quantities of malondialdehyde (MDA) and 1- diphenyl- 2-picrylhydrazyl (DPPH). The thiobarbituric acid reactive substances (TBARS) test was conducted following the methods of Sarmiento-García et al. (2021) and Kilic & Richards (2003) to determine the MDA value. The DPPH radical scavenging activity was determined as described by Sacchetti et al. (2005) and Olgun et al. (2022) with slight modifications. And was used to evaluate the antioxidant capacity of the produced hydrolysates.

The mineral contents of faeces and tibia samples have been evaluated using the wet digestion technique. All analysis for faeces and tibia mineral contents were conducted according to the methods described by Gül et al. (2024). On the 84th day, one quail from each subgroup (n = 25) was randomly chosen and euthanized via cervical dislocation. The right tibia was extracted, cleaned, and stored at -20°C until further analysis. Before examination, the samples were brought to room temperature for six hours in a controlled air environment. The biomechanical properties of the bones were evaluated following the methodologies of Wilson & Ruszler (1996), Armstrong et al. (2002), and Gül et al. (2022).

The statistical analysis utilized cage averages as the experimental units for overall comparisons, with a one-way ANOVA conducted via SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). For specific parameters such as egg quality, MDA, and DPPH levels, individual birds served as the experimental units. The findings are reported as means ± standard errors of the mean (SEM). Statistical significance was established at $P<0.05$. The response of each dependent variable to increasing manganese levels was evaluated using linear, quadratic, and cubic regression models.

RESULTS AND DISCUSSIONS

The National Research Council (1994) established the Mn requirements for avian species to be 60 mg/kg. Since this data was published in 1994, significant advancements in animal production, primarily due to genetic improvements, have occurred. These changes have enhanced production rates and consequently altered the nutritional requirements of animals. Therefore, it is necessary to re-evaluate these nutritional needs to reflect the current state of animal production (Jasek et al., 2019; Gül et al., 2023).

Performance and egg indicators are detailed in Table 2. MnH supplementation appeared to have minimal impact (P>0.05) on final body weight (270.6-274.1 g), body weight change (10.75-20.13 g), egg weight (12.62-13.26 g), and feed conversion ratio (2.73-2.93), as no significant differences were detected between treatments. Egg production was significantly lower (87.19 per egg/100 quails) in the group without MnH supplementation compared to the other experimental diets (89.85-90.92 though the differences between the MnH-supplemented groups were not statistically significant (P>0.05). This pattern was also observed in feed intake, where MnH supplementation led to a significant decrease (P<0.05). However, feed intake values were similar (P>0.05) among all groups that received MnH supplementation. The current research found that MnH supplementation does not affect performance (in terms of final body weight, body weight change, and feed conversion ratio) compared to non-supplemented quails.

Table 2. Influence of dietary MnH levels on the performance of laying quails (n = 125)

Parameters	MnH levels (mg/kg)					S.E.M*	*p*-values	Regression		
	18.86	40	60	80	100			L	Q	C
Initial BW (g)	252.2	254.0	262.8	261.6	254.1	2.35	0.518	0.492	0.180	0.405
Final BW (g)	270.6	274.1	273.5	270.9	273.1	3.67	0.964	0.882	0.555	0.819
BWC (g)	18.48	20.13	10.75	14.88	16.75	1.828	0.569	0.515	0.408	0.475
EP (per egg/100 quails)	87.19[b]	89.85[a]	90.18[a]	90.19[a]	90.92[a]	0.422	0.037	0.006	0.189	0.276
EW (g)	12.75	12.62	13.26	12.95	13.08	0.129	0.572	0.304	0.724	0.698
EM (g/quail/day)	11.12	11.34	11.95	11.68	11.89	0.125	0.155	0.033	0.392	0.968
FI (g/quail/day)	31.29[b]	33.18[a]	32.63[a]	33.28[a]	33.45[a]	1.186	0.012	0.003	0.178	0.172
FCR	2.83	2.93	2.73	2.85	2.82	0.024	0.137	0.496	0.855	0.334

S.E.M.: Standard error of means, L: Linear, Q: Quadratic, C: Cubic, [a,b]: At the P < 0.05 level, means in a row with distinct upper letters vary from one another. BW: Body weight, BWC: Body weight change, EP: Egg production, EW: Egg weight, EM: Egg mass, FI: Feed intake, FCR: Feed conversion ratio.

Similar findings have been reported by previous authors in laying hens (Bai et al., 2014; Xiao et al., 2014; Hajjarmanesh et al., 2023) and broilers (Jasek et al., 2019; Saldanha et al., 2020). As described by Jasek et al. (2019), the absence of the noted differences in performance parameters by rising Mn inclusions could suggest: i) the weight increase demand is rather weak and can be met by Mn levels in dietary ingredients, ii) the enzymes influenced by Mn are not related to increased body weight gain, or iii) a combination of both. However, higher feed intake was reported for all supplemented groups compared to the non-supplemented. In contrast, no differences in this parameter have been found in previous studies on broilers (Jasek et al., 2019; Groff-Urayama et al., 2020), while Khoshbin et al. (2023) showed a trend of increased feed intake with the addition of organic Mn chelate to the diet of laying hens, similar to the findings in this study. Jasek et al. (2019) described that the mechanisms regulating appetite might not be related to Mn intake and could depend on the intake of other minerals. This observation differs from what has been noted in our study for laying quails. Since there is limited information on this subject, this parameter should be re-evaluated to confirm possible differences between quails, broilers, and laying hens. In the current research, egg production rose significantly with increased Mn in the diet, similar to findings by previous authors for laying hens (Noetzold et al., 2020; Khosbin et al., 2023) and ducks (Zhang et al., 2022). For example, reductions in circulating progesterone, estradiol, FSH, and LH are suggestive of Mn deficit, which may impair the function of the hypothalamic-pituitary gonadal axis. Additionally, declines in the activity of certain enzymes (such as pyruvate carboxylase) up-regulated by Mn are associated with lower egg production (Noetzold et al., 2020; Zhang et al., 2022). These outputs align with the recommendation of the National Research Council (1994) which proposed that diets for laying quail should contain at least 60 mg/kg Mn to maintain optimal quail developmental and egg production parameters.

Eggshell quality remains a critical issue for avian production due to its significant economic implications for producers (Gül et al., 2022; Hajjarmanesh et al., 2023). Table 3 illustrates the impact of varying dietary MnH levels on eggshell quality. The incidence of damaged eggs (ranging from 0.16 to 2.21 per 100 eggs) did not show significant variation with different MnH supplementation levels (P>0.05). However, quails receiving 80 mg/kg (13.25 N) and 100 mg/kg (13.03 N) Mn exhibited significantly greater eggshell strength (P<0.01) compared to those in the non-supplemented group and those receiving 40 mg/kg and 60 mg/kg Mn (11.78 N, 11.62 N, and 11.60 N, respectively). In terms of relative eggshell weight, the group supplemented with 80 mg/kg MnH had the highest value (9.22 g shell/100 g egg), significantly surpassing the non-supplemented group and those supplemented with 40 and 60 mg/kg (8.19, 8.43, and 8.15 g shell/100 g egg, respectively (P<0.01). Additionally, eggshell thickness was notably higher (P<0.01) in the group receiving 80 mg/kg Mn (244.8 µm) compared to the control groups and the group receiving 60 mg/kg MnH (229.9 µm and 232.7 µm, respectively). In this study, the thickness, breaking strength, and weight of eggshells have significantly been enhanced at levels of 80 mg/kg MnH compared to non-supplemented quails. These findings align with those of Noetzold et al. (2020) and Hajjarmanesh et al. (2023), who reported similar improvements when manganese was added to the diets of laying hens. Cui et al. (2019) observed that adding 21.95 mg/kg of Mn to a basal diet enhanced eggshell thickness and breaking strength, and supplementing with 40 mg/kg Mn was sufficient for optimal shell quality. In another study, Xiao et al. (2014) described that a dietary addition of 100 mg/kg Mn to the diet enhanced shell thickness and breaking strength.

Table 3. Influence of dietary MnH levels on the eggshell quality (n = 300) of laying quails

Parameters	MnH levels (mg/kg)					S.E.M*	*p*-values	Regression		
	18.86	40	60	80	100			L	Q	C
Damaged egg rate (per egg/100 eggs)	0.89	1.01	0.17	2.21	0.16	0.275	0.101	0.883	0.515	0.099
Eggshell-breaking strength (kg)	11.78^b	11.62^b	11.60^b	13.25^a	13.03^a	0.204	0.003	0.001	0.207	0.084
Relative eggshell weight (g shell/ 100g egg)	8.19^c	8.43^{bc}	8.15^c	9.22^a	8.75^{ab}	0.104	0.001	0.001	0.972	0.065
Eggshell thickness (µm)	229.9^b	238.2^{ab}	232.7^b	244.8^a	241.7^a	1.62	0.008	0.003	0.649	0.897

S.E.M.: Standard error of means, L: Linear, Q: Quadratic, C: Cubic, ^{a,b}: At the P < 0.05 level, means in a row with distinct upper letters vary from one another.

Zarghi et al. (2023) observed that shell weight increased in laying hens receiving Mn at a level of 0-90 mg/kg, while other eggshell quality criteria have been unaffected. The results of this study suggest that the MnH level required to improve eggshell quality parameters is higher than the level needed to maintain overall performance and productivity in laying quails. The observed improvements in eggshell quality (increased breaking strength, thickness, and relative weight) as dietary Mn increased are likely due to the enhancement of the palisade and mammillary layers, along with the shell membrane. Mn is essential for the synthesis of glycosaminoglycans and glycoproteins, as it regulates glycosyltransferase, an enzyme involved in the production of proteoglycans, which are key components of the eggshell matrix. Consequently, Mn supplementation has been associated with increased membrane glycosaminoglycan content, which contributes to improved shell morphology (Noetzold et al., 2020).

Mn is an integral part of manganese superoxide dismutase (Mn-SOD), the primary antioxidant enzyme that protects cells from oxidative stress, neutralizes reactive oxygen species, reduces lipid peroxidation, and enhances overall antioxidant capacity (Zhu et al., 2017). According to the data in Table 4, the yolk TBARS value (1.414-2.230 µmol MDA/kg) has been unaffected by dietary MnH levels (P>0.05). However, according to these results, the DPPH value could be more sensitive to MnH rose in comparison to those described for TBARS value. The highest dose of MnH (at a level of 100 mg/kg Mn) resulted in a significant rise (P<0.01) in the DPPH value compared to the rest of the experimental groups.

Table 4. Influence of dietary MnH levels on the yolk (n = 100) DPPH and MDA values of laying quails

Parameters	MnH levels (mg/kg)					S.E.M*	*p* -values	Regression		
	18.86	40	60	80	100			L	Q	C
DPPH (% reducing)	6.764^{b}	6.857^{b}	6.154^{b}	6.936^{b}	9.125^{a}	0.2017	<0.001	<0.001	<0.001	0.029
MDA (μmol MDA/kg)	1.604	2.230	1.958	1.782	1.414	0.1349	0.368	0.408	0.091	0.481

S.E.M.: Standard error of means, L: Linear, Q: Quadratic, C: Cubic, ^{a,b}: At the P < 0.05 level, means in a row with distinct upper letters vary from one another. TBARS: thiobarbituric acid reactive substances, DPPH: 2,2-diphenyl-1-picrylhydrazyl.

In this study, the reduction percentage of DPPH in the yolk increased significantly at a dietary level of 100 mg/kg MnH, while other groups, including the non-supplemented group, exhibited similar DPPH values. These outputs suggest an adequate bioavailability of Mn at doses of 100 mg/kg that would be transported and deposited into the yolk to exert its antioxidant capacity. This is linked to the rise in Mn-SOD activity and the decrease in lipoprotein lipase activity with higher Mn supplementation. Mn-SOD increases antioxidant capacity to scavenge reactive oxygen species and decline lipid peroxidation as can be observed in this study (Cui et al., 2019; Khosbin et al., 2013). Nevertheless, the yolk MDA value has been unaffected by MnH supplementation. To our knowledge, there are no studies that jointly examine the effect of MnH on DPPH and TBARS values in egg yolk. Nevertheless, numerous studies have demonstrated the antioxidant benefits of dietary Mn supplements in various tissues. Khoshbin et al. (2023) clarified that the Mn addition to the diet at a level of 90 mg/kg enhanced the serum DPPH and declined MDA values in laying hens. Similar findings have been obtained in broilers, Bozkurt et al. (2015), described a decrease in serum MDA levels with dietary Mn ranging from 6.25 to 50 mg/kg. Additionally, Mn supplementation has been shown to effectively reduce lipid oxidation in broilers (Shokri et al., 2021) and duck meat (Yang et al., 2021).

Mn that is not absorbed in the intestine is excreted in the bile or faeces (Zhang et al., 2022), so as expected, as the dietary Mn level increased, the faecal Mn content also increased. Table 5 summarises the impact of different dietary levels of MnH on the mineral content in quail faeces. There was a significant positive correlation (P<0.001) between the levels of MnH in the diet and the concentration of Mn in the faeces Specifically, quails on an Mn-deficient diet exhibited the lowest faecal Mn levels, while those receiving higher Mn supplements showed increased excretion. Interestingly, the dietary Mn level did not significantly influence faecal zinc content (P>0.05), though a quadratic regression trend (P<0.05) was noted. Conversely, faecal copper content declined linearly (P<0.01) with increasing Mn supplementation, dropping from 27.26 mg/kg in the non-supplemented group to 21.94 mg/kg in the group receiving 100 mg/kg of Mn. Calcium and phosphorus excretion patterns also responded to Mn supplementation.

Table 5. Influence of dietary MnH levels on the faeces mineral composition (n = 25) of laying quails

Parameters	MnH levels (mg/kg)					S.E.M*	*p*-values	Regression		
	18.86	40	60	80	100			L	Q	C
Manganese (mg/kg)	199.8^{d}	212.9^{cd}	223.9^{c}	249.4^{b}	269.0^{a}	5.57	<0.001	<0.001	0.147	0.809
Zinc (mg/kg)	313.3	324.7	327.1	320.0	297.3	4.13	0.144	0.205	0.024	0.762
Copper (mg/kg)	27.26^{a}	25.07^{b}	23.40^{bc}	24.62^{b}	21.94^{c}	0.457	<0.001	<0.001	0.486	0.067
Calcium (g/100g)	5.04	6.27	4.90	5.18	5.26	0.173	0.080	0.582	0.561	0.038
Phosphorus (g/100g)	1.56	1.85	1.79	1.87	1.93	0.045	0.063	0.012	0.344	0.248

S.E.M.: Standard error of means, L: Linear, Q: Quadratic, C: Cubic, ^{a,b}: At the P < 0.05 level, means in a row with distinct upper letters vary from one another.

A cubic regression (P<0.05) was evident for calcium, with the highest excretion noted in the 40 mg/kg Mn group. Phosphorus excretion showed a linear regression (P<0.05), peaking at the 100 mg/kg Mn supplementation level. Similar to the current study, Mwangi et al. (2019), Matuszewski et al. (2020), de Carvalho et al. (2021), Xia et al. (2022) and Zhang et al. (2022) reported that the Mn level excreted through faeces increased with the increasing Mn in the diet in avian. Nevertheless, it is important to point out that regardless of the doses, the current findings reveal a lower mineral excretion than those reported when inorganic Mn has been added to the avian´s diet. This is due to the increased absorption of organic forms such as hydroxychloride, reducing the excretion through the faeces and contributing to environmental protection (Jasek et al., 2020; Jiang et al., 2021; Groff-Urayama et al., 2023; Olgun et al., 2024). Table 6 shows the impact of different levels of dietary MnH on the biomechanical properties of the tibia in laying quails.

Cortical bone thickness, shear force, cortical bone cross-sectional area, and shear stress have significantly been affected by the treatments ($P<0.01$). Cortical bone cross-sectional area and cortical bone thickness showed similar behaviour, with a high correlation with increasing concentration of MnH ($P<0.01$). Contrary to the expected, both parameters decreased significantly as the concentration of MnH rose (from 0.476 to 0.331 mm and from 1.79 to 1.27 mm^2, respectively). Shear force and shear stress parameters decreased with the addition of MnH to the diet (from 187.2 to 115.5 N and from 106.57 to 80.45 N/mm^2, respectively).

The shear force recorded the lowest values with a dietary dose of Mn of 60 mg/kg, while shear stress showed the lowest values in the groups that had received 40 and 60 mg/kg of MnH in the diet. It has been described that Mn regulates the formation of proteoglycans, which are involved in bone development (Jasek et al., 2019). Contrary to those expected, the outputs of the current research do not seem clear. The biomechanical traits of the tibia in laying quails that had been supplemented with MnH have been observed to be worse compared to the non-supplemented group. Similar findings have been reported by Jasek et al. (2019) who found inconsistent findings with the supplementation of high doses of Mn. Those authors considered that the outputs are inconclusive and proposed that the Mn levels evaluated are not sufficient to improve bone parameters, and it would be necessary to use a larger number of birds in future experiments to evaluate these parameters. According to the previous literature, studies assessing bone parameters are scarce and contradictory. Cui et al. (2019) demonstrated that the administration of Mn (0-800 mg/kg) to the diet does not affect the tibia-breaking strength, similar to those reported by Bozkurt et al. (2015) and Shokri et al. (2021), who described that the inorganic or organic Mn addition had no impact on the tibia biomechanical properties of broilers. The duration of the study, the age of the animals and the species used may be responsible for the variations observed between previous studies. Future research would be necessary to confirm the findings obtained for the laying quails.

The tibia mineral level of laying quails is reflected in Table 7. Tibia mineral content has not been seen to be sensitive to MnH supplementation. No differences ($P > 0.05$) in the tibia mineral content have been described amongst experimental groups for any of the minerals assessed. The minimum and maximum values of the tibia mineral levels are as follows: Mn (12.78-10.22 mg/kg), zinc (311.6-289.8 mg/kg), copper (3.67-2.83 mg/kg), calcium (25.11-22.78 g/100 g) and phosphorus (18.32-16.11 g/100 g). Contrary to expectations, but following the outputs obtained in the tibia parameters, MnH supplementation does not affect the mineral concentration of the tibia, which is consistent with those proposed by Xiao et al. (2015). Similarly, Bai et al. (2014) explained that the supplementation of 60 and 300 mg/kg Mn to the basal diet (24.35 mg/kg Mn) in hens had no effect on tibia zinc and copper content but raised tibia Mn level (300 mg/kg Mn). On the opposite, Yıldız et al. (2011) reported that the Mn addition to the basal diet in laying hens improved the tibia Mn, calcium, and phosphorus however caused a decrease in zinc and copper.

In another study, Cui et al. (2019) stated that Mn addition to the diet enhanced the Mn level while not affecting the tibia calcium content, similar to those described by Jasek et al. (2019). Several explanations may be responsible for the differences found among the different studies. For example, Junchang & Ruangpanit (2023), who examined the concentration of some minerals in bones from old hens, reported that old hens are less sensitive to Mn administration, which could justify the findings of the current research. In addition, the species used could be responsible for the differences. Regardless, more investigation is required to reassess these findings.

Table 6. Influence of dietary MnH levels on the tibia biomechanical traits (n = 25) of laying quails

Parameters	MnH levels (mg/kg)					S.E.M*	*p*-values	Regression		
	18.86	40	60	80	100			L	Q	C
Cortical bone thickness (mm)	0.476[a]	0.410[bc]	0.383[cd]	0.455[ab]	0.331[d]	0.0134	<0.001	0.001	0.865	0.002
Cortical bone cross-sectional area (mm^2)	1.79[a]	1.55[b]	1.43[c]	1.56[b]	1.27[c]	0.046	0.001	<0.001	0.615	0.035
Shear force (N)	187.2[a]	131.0[bc]	115.5[c]	150.9[b]	129.7[bc]	6.15	<0.001	0.002	0.001	0.003
Shear stress (N/mm^2)	106.57[a]	84.67[c]	80.45[c]	96.75[ab]	102.11[a]	2.876	0.005	0.906	0.001	0.109

S.E.M.: Standard error of means, L: Linear, Q: Quadratic, C: Cubic, [a,b]: At the P < 0.05 level, means in a row with distinct upper letters vary from one another.

Table 7. Influence of dietary Mn levels on the tibia mineral composition of laying quails (n = 25)

Parameters	Mn levels (mg/kg)					S.E.M*	*p*-values	Regression		
	18.86	40	60	80	100			L	Q	C
Manganese (mg/kg)	12.78	10.22	11.48	10.51	11.33	1.538	0.058	0.170	0.056	0.314
Zinc (mg/kg)	311.6	289.9	294.0	297.6	289.8	7.62	0.910	0.531	0.687	0.536
Copper (mg/kg)	3.18	3.32	2.83	2.90	3.67	0.135	0.280	0.566	0.117	0.145
Calcium (g/100g)	24.67	23.71	25.11	24.91	22.78	1.793	0.191	0.262	0.215	0.076
Phosphorus (g/100g)	17.75	16.11	17.37	18.32	17.09	0.355	0.389	0.739	0.809	0.055

S.E.M.: Standard error of means, L: Linear, Q: Quadratic, C: Cubic, [a,b]: At the P < 0.05 level, means in a row with distinct upper letters vary from one another.

CONCLUSIONS

This study offers a significant advancement in understanding the effects of MnH supplementation in laying quails. The findings indicate that MnH supplementation is beneficial for enhancing certain performance indicators, egg production, and egg quality in laying quails, with 80 mg/kg MnH identified as a particularly effective dose. However, the effects on bone integrity remain unclear. Regarding environmental impact, while increased MnH doses resulted in higher Mn excretion, the levels were still lower than those recorded for other sources of organic Mn. This suggests that MnH is a more environmentally friendly option compared to other Mn sources. Nevertheless, the beneficial effects on bone integrity are not clear. Further research is necessary to fully understand the implications of MnH supplementation on avian bone health and to explore the long-term effects of MnH supplementation and its interaction with other dietary components.

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EVALUATION OF THE HEALTH STATUS OF DAIRY COWS DURING A MYCOTOXIN SCREENING OF FEED IN A FARM FROM NORTH-EAST ROMANIA

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Abstract

The purpose of this study was to analyze the health status of dairy cows from a farm in Moldova. A total of 30 feed samples were analyzed from a mycotoxicological point of view, and 90 blood samples were collected from cattle of the BNR breed, which were biochemically analyzed to investigate the state of health. Adverse effects of mycotoxins are manifested in health, production, and reproduction in ruminants, especially dairy cows, and in the population. During the experimental period, regarding mycotoxin contamination of feed, the levels of mycotoxins investigated (AFL-T, FUM, and DON) did not exceed legal limits. From a biochemical point of view, the parameters that did not fall within the limits of the reference interval and showed a slight increase were represented by ALT, Gamma GT, and urea. All cows involved in the study were apparently healthy at the time of sample collection, but the resulting biochemical analyses suggest possible mild liver disease (by increasing above the normal limits of the previously mentioned parameters).

Key words*: biochemical analyses, bovine serum, dairy cows, mycotoxins.*

INTRODUCTION

Mycotoxins are by-products like antibiotics, only mycotoxins are toxic to animals and humans. Due to their high toxicity, even minute levels can have an impact on an individual's health (Alassane-Kpembi et al., 2016). According to Alonso et al. (2015), nearly all mycotoxins are cytotoxic, capable of rupturing cell membranes and influencing or preventing the creation of DNA, RNA, and proteins. However not every fungal growth produces mycotoxins, and finding fungi does not always mean that mycotoxins are present. Eating a diet tainted with mycotoxins can have both short-term and long-term consequences, such as immune system suppression, teratogenic, carcinogenic, and estrogenic effects (Cheli et al., 2013). Concurrent exposure to numerous mycotoxins is more common in the livestock business, despite the fact that the scientific literature offers a wide range of knowledge regarding the effects of specific mycotoxins on various animal species (Chukwudi et al., 2021). The reported illness signs and/or poor animal performance in commercial operations could be the result of numerous mycotoxins interacting synergistically. Some mycotoxins can enter milk and have negative effects not just on animal health but also on food safety and human health (Gallo et al., 2015; Sirbu et al., 2020). Only aflatoxin has been reported to be transmitted into the milk of nursing cattle at significantly elevated levels of concern out of all the mycotoxins examined (Penagos-Tabares et al., 2021). It is not always the case that fungal contamination is the cause of an animal's illness, as fungi can exist without creating mycotoxins. Because the mycotoxicological, clinical, radiological, microbiological, and histological data alone cannot provide the information required for the correct diagnosis, these data must be corroborated in order to produce an accurate diagnosis. According to Solfrizzo et al. (2014), the effects of mycotoxin ingestion are primarily chronic and involve concealed problems with decreased intake, productivity, and fertility.

Through clinically confusing changes in animal husbandry, decreased food consumption or refusal of feed, altered nutritional absorption and metabolism, effects on the endocrine system, and immune system suppression, these consequences result in significant economic losses (Stoev, 2015). In order to maintain the state of health and obtain production at optimal or even good levels, it is necessary to observe the control regarding the quality of feed, thus the periodic mycotoxicological analysis of the feed is an important way of assessing their quality both for their administration in the nutrition of dairy cows and for the preservation process (Coroian et al., 2017).
The health status of cows can be assessed and depends on the biochemical profile of the blood. If the physiological limits are normal, the biochemical profile reflects a good state of health, being also correlated with milk production (Magdaléna et al., 2020).

MATERIALS AND METHODS

The material is represented by the 30 samples, such as corn grains (15) and mixed fodder ration (15) from a cattle farm whose study the article refers to. Only these feed categories (corn grain and mixed fodder ration) were chosen because they were considered to be the most relevant and important. The structure of the mixed fodder ration for the dairy cow includes corn, triticale, soybean sorghum, rapeseed sorghum, mixed fodder premix, brewer's yeast, etc. The samples were collected on different days and months, namely March-May, respectively June-August 2023. The mycotoxicological analysis of the fodder was carried out by the ELISA method, the method used for the quantitative determination of mycotoxins. The basic principle is the antigen-antibody reaction, and the mycotoxins are detected by means of the color reaction, the intensity of which is measured by means of the ELISA immunoenzymatic line.
The short working protocol within the ELISA technique involves:
a) adding 200 µL of the conjugated solution to each color-coded dilution well;
b) adding 100 µL of each standard or sample to the dilution wells containing 200 µL of conjugate;
c) mix well, then immediately transfer 100 µl of the contents of each dilution well into a microwell coated with appropriate antibodies and incubate at room temperature for 15 minutes;
d) the contents of the microwells are thrown away and wash the wells with deionized water or buffer solution (5x);
e) dry the wells on an absorbent paper towel;
f) add 100 µl of the substrate solution to each well and incubate for 5-20 minutes;
g) add 100 µl of stop solution to each well;
h) read the absorbance of each well within 10 minutes of the addition of the stop solution at 450 nm (reference wavelength 630 nm) with a microwell reader.
Mycotoxins are extracted from a ground sample (20 g) with 70% methanol, only deoxynivalenol is extracted from a ground sample with distilled water. The extracted sample and enzyme-conjugated mycotoxins are then mixed and added to the antibody-coated microwell. The mycotoxins in the samples are allowed to complement the enzyme-conjugated mycotoxin at the antibody binding sites. After a washing step, the enzyme substrate is added, which results in color development. The intensity of the color is inversely proportional to the mycotoxin concentration in the sample. A stop solution is then added which changes the color from blue to yellow and the absorbance of each well is then measured at 450 nm. The kits provided by Romer Labs were used to analyze the content of total aflatoxins, fumonisins, and deoxynivalenol in the samples, kits that comply with the specifications of EN ISO 9001 and EN ISO 17025 respectively. The working technique refers to the protocol recommended by the manufacturer of the AgraQuant kit.

Figure 1. ELISA immunoenzymatic line (Tecan, Hydro-Flex model) (own source)

The analysis is carried out to identify metabolic disorders in an animal, and lack of nutrients in the body. Ninty blood samples were collected and analyzed from cows of the BNR breed during 6 months (March-August). The cows were divided into three groups, depending on milk production, thus group G1 assumes a milk production of 31 liters, group G2 assumes a milk production of 28 liters and group G3 assumes a milk production of 16 liters/day. After collection, blood samples were centrifuged for 5 minutes at 3000 rpm in a Hettich Zentrifugen Rotofix 32A Centrifuge. The resulting supernatant is called serum. After centrifugation, it is important that the liquid component (serum) is immediately transferred to a graduated, capped Eppendorf tube using a pipette. If the serum is not analyzed immediately, the serum should be stored and transported at -20°C or less. Freeze-thaw cycles are important to avoid, as this is detrimental to many serum components. Blood sera were analyzed for the following biochemical parameters: ALT/GPT (Alanine aminotransferase), ALB (albumin), AST/GOT (Aspartate aminotransferase), TBIL (total bilirubin), CA (calcium), CHOL (cholesterol), CREA (creatinine), gamma-Gt, TP (total protein), TRIG (triglycerides), BUN (urea) and ALKP (alkaline phosphatase). Analyzes were determined using the BioSystems BA200 analyzer, which features a dynamic baseline with SMART LED technology (Figure 2).

Figure 2. BioSystems BA200 analyzer (own source)

Among other things, this analyzer offers the highest degree of flexibility, the dosing is very accurate and it is a compact system with low maintenance. It has a large reagent and sample loading capacity. The values of the biochemical parameters in the serum obtained from the analyses were statistically evaluated by SPSS21. The ANOVA test coupled with the LSD test was performed to highlight the differences in the parameters during the studied period.

RESULTS AND DISCUSSIONS

The most important mycotoxins that contaminate corn and derived products during development and storage are total aflatoxins (AFL-T), fumonisins (FUM), deoxynivalenol (DON), and zearalenone (ZEA) (Table 1).

In the present work, the feed samples were mycotoxicologically analyzed only with regard to the first three mycotoxins previously mentioned.

Aflatoxin is a mycotoxin produced by the species *A. flavus* and *A. parasiticus*. As the most potent natural liver carcinogen known, aflatoxin causes thousands of cases of liver cancer annually worldwide (Jina Wu et al., 2022). Aflatoxin is the only mycotoxin on which the FDA has placed limitations on how much can be found in dairy rations (May et al., 2000). Fumonisins are produced by *F. verticillioides* and *F. proliferatum* plus other species of fungi (Mannaa & Kim, 2007). Fumonisins are often detected in corn silage and can cause important productivity losses but also health problems in both beef and dairy cattle. Even though ruminants are still considered less sensitive to fumonisins, there is evidence that fumonisins, alone or in combination with other *Fusarium* mycotoxins, have a hepatotoxic effect and affect immune functions in ruminants (Chulze, 2010). Deoxynivalenol is also called vomitoxin and is produced by species of the *Fusarium* genus. The mycotoxin deoxynivalenol is among the least toxic of the trichothecenes, and its effects are common. It should be noted that in combination with other mycotoxins (fumonisins, T_2 toxin) it can have much more serious effects (Udovicki et al., 2018). The study regarding the presence of total aflatoxins, fumonisins, and deoxynivalenol was carried out on 15 samples of corn grains and 15 samples of mixed fodder ration, as presented in Table 1. Also, in Tables 2 and 3 the statistical estimators of the main samples are presented of fodder studied (corn grains and mixed fodder ration), respectively the maximum limits allowed.

Table 1. The results of the mycotoxicological examination of the analyzed feed samples

The analysis period	Sample	AFL-T Total aflatoxins (µg/kg)	FUM - Fumonisins (µg/kg)	DON- Deoxynivalenol (µg/kg)
March-May	Corn grains	4.66	255.16	819.35
March-May	Corn grains	3.15	317.11	982.71
March-May	Corn grains	4.25	114.8	1108.5
March-May	Corn grains	6.84	195.3	986
March-May	Corn grains	2.11	266.2	1117.36
March-May	Corn grains	4.30	178.16	892.4
March-May	Corn grains	3.68	418.22	788.31
June-August	Corn grains	6.30	542	1144.5
June-August	Corn grains	4.77	314.2	799
June-August	Corn grains	3.14	351	412.33
June-August	Corn grains	6.72	216.5	652.81
June-August	Corn grains	6.47	180.11	839.99
June-August	Corn grains	5.22	237.66	1087
June-August	Corn grains	4.16	167.2	960.5
June-August	Corn grains	3.95	206.8	1116.1
March-May	Mixed fodder ration	0.11	115	1002.4
March-May	Mixed fodder ration	0.05	198.4	716.55
March-May	Mixed fodder ration	0.01	94.2	608.1
March-May	Mixed fodder ration	0.63	131.4	422.83
March-May	Mixed fodder ration	0.01	188.9	315.22
March-May	Mixed fodder ration	1.20	156.28	414
March-May	Mixed fodder ration	0.83	60.9	262.95
June-August	Mixed fodder ration	1.45	124.1	212
June-August	Mixed fodder ration	0.05	210.88	320.4
June-August	Mixed fodder ration	0.96	131	412.33
June-August	Mixed fodder ration	0.08	96.3	247.01
June-August	Mixed fodder ration	0.55	168.66	188.6
June-August	Mixed fodder ration	1.76	214.7	308.6
June-August	Mixed fodder ration	1.82	140.62	254
June-August	Mixed fodder ration	1.09	110.83	372.98

Table 2. Statistical estimators - corn grains

Mycotoxicological parameters	Standard limit *(REGULATION (EC) NO. 1881/2006)*	Statistical estimators - corn grains				
		$\bar{X}$	s	CV, %	Min.	Max.
Total aflatoxins (µg/kg)	10	4.65	1.42	0.31	2.11	6.84
Fumonisins (µg/kg)	4000	264.03	264.03	0.42	114.8	542
Deoxynivalenol (µg/kg)	1750	913.79	203.36	0.22	412.33	1144.5

Table 3. Statistical estimators - mixed fodder ration

Mycotoxicological parameters	Standard limit *(REGULATION (EC) NO. 1881/2006)*	Statistical estimators - mixed fodder ration				
		$\bar{X}$	s	CV, %	Min.	Max.
Total aflatoxins (µg/kg)	4	0.71	0.65	0.93	0.01	1.82
Fumonisins (µg/kg)	4000	142.81	45.92	0.32	60.9	214.7
Deoxynivalenol (µg/kg)	1750	403.86	219.28	0.54	188.6	1002.4

Analyzing the results presented in Tables 1, 2, and 3, we can note that the determined values do not exceed the limits established by Regulation (EC) no. 1881/2006, thus for corn grains, the maximum allowed limit regarding total aflatoxins is a maximum of 10 µg/kg, while for the mixed fodder ration the maximum allowed limit is 4 µg/kg. The average value obtained for corn grains, in the case of total aflatoxins, was 4.65 µg/kg, while for the mixed fodder ration, it was 0.71 µg/kg, values according to the standard.
The level of fumonisins allowed in feeds represented by corn and derived from corn, according to Regulation (EC) no. 1881/2006, is 4000 µg/kg. From the samples analyzed in this study, it emerged that the average obtained fell within the limit allowed by the standard: corn grains - 264.93 µg/kg, respectively mixed fodder ration - 142.81 µg/kg.
For the deoxynivalenol parameter, the specialized literature shows that the determined values of samples from Romania, compared to other studies from other parts of the world, are low. In the present case, the determined values of the analyzed feed samples did not exceed the maximum established limit of 1750 µg/kg according to the results obtained: corn grains - 913.79 µg/kg, respectively mixed fodder ration - 403.86 µg/kg (Tables 1, 2 and 3).
Even if the results obtained were in accordance with the specialized literature and fell within the maximum limits allowed by the standard, from a percentage point of view, deoxynivalenol had a higher level of contamination in the 30 feed samples (77.30% - corn grains, respectively 73.78% - mixed fodder ration) (Table 4, Figure 3).

Table 4. Obtaining the percentage of mycotoxins in the analyzed samples

Mycotoxins	Corn grains		Mixed fodder ration	
	Average (n=15)	Mycotoxin percentage (%)	Average (n=15)	Mycotoxin percentage (%)
AFLA-T (µg/kg)	4.32	**0.37**	0.71	**0.13**
FUM (µg/kg)	264.03	**22.33**	142.81	**26.09**
DON (µg/kg)	913.79	**77.30**	403.86	**73.78**

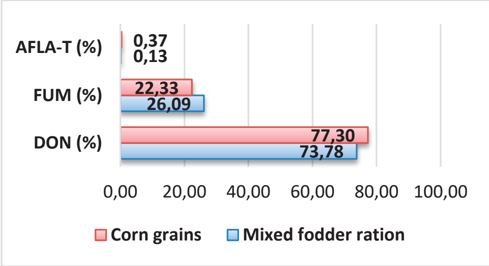

Figure 3. The percentage of mycotoxins in the analyzed samples

In second place was the class of fumonisins, in which only 22.33% were present in corn grains and 26.09% in mixed fodder ration (Table 4, Figure 3). In third place were the total aflatoxins, which represented the lowest level of contamination, such as 0.37% (corn grains) and 0.13% (mixed fodder ration) (Table 4, Figure 3).
Between the two categories of fodder, corn kernels presented a higher concentration of mycotoxins in contrast to the mixed fodder ration, with the mention that they were within legal limits. According to the literature, the negative impact of fumonisins in cattle is observed only when the animals are exposed to extremely high levels of mycotoxin, such as 90 mg/kg, levels that are much higher than the maximum recommended levels. The presence of fumonisins does not cause damage to ruminants (Antonio, 2022).
The degradation of deoxynivalenol depends a lot on the functions of the rumen, therefore chronic exposure should not be neglected. Thus, it is known that this mycotoxin - deoxynivalenol can produce inflammatory processes, even if its presence in the diet is below the maximum recommended levels (Gallo et al., 2020). The adverse effects of mycotoxins are manifested both in health, production, and reproduction in ruminants, especially dairy cows, and in the human milk-consuming population.
A biochemical profile is recommended whenever necessary because biochemical parameters change their values, which is characteristic of certain pathological conditions. The serum samples were analyzed from a biochemical point of view, and the average of the parameters, respectively the non-conforming average values obtained are in Table 5.

Table 5. The average of the biochemical parameters for each month

Biochemical Parameters	Reference interval *(The Merck Veterinary manual)*	The average of the parameters ($\bar{X}$) / Month					
		March	April	May	June	July	August
Albumin (g/L)	29-39	34.87	33.87	29.60	34.27	33.47	34.13
ALP-DEA (U/L)	27-127	87.33	93.33	87.60	74.67	120.47	122.87
ALT-GPT(U/L)	5-18	24.80	24.67	23.07	24.13	27.27	25.93
AST-GOT(U/L)	60-125	102.27	104.93	95.60	109.80	84.73	78.13
Total bilirubin(mg/dL)	0-1.6	0.08	0.09	0.05	0.08	0.23	0.08
Calcium (mg/dL)	8-11.4	8.46	8.46	7.48	8.39	8.54	8.34
Cholesterol(mg/dL)	163-397	201.73	216.53	180.87	192.27	196.33	175.13
Creatinine(mg/dL)	0.5-2.2	0.71	0.68	0.69	0.75	0.56	0.57
Gamma-GT(U/L)	6-17.4	34.20	37.80	25.00	37.87	25.20	26.67
Total protein(g/L)	59-81	71.67	74.80	67.80	82.93	75.33	79.27
Triglycerides (mg/dL)	10-19	6.94	5.14	2.80	14.47	18.27	16.67
Urea(mg/dL)	10-25	35.80	34.00	27.53	22.53	8.87	12.40

In March, the biochemical parameters that did not fall within the limits and showed a significant increase were: ALT-GPT, Gamma-Gt, and urea. Only the triglyceride content was lower than the maximum provided by the specialized literature. In April, the results of biochemical parameters are similar to March. Slight-moderate increases in ALT-GPT, Gamma-Gt, and urea can indicate diseases with liver location and not only. The reduced content of triglycerides demonstrated the fact that the animals were in a negative energy balance. Regarding the month of May, all the parameters fell within the stipulated limits, with the exception of 5 of them, thus ALT, Gamma, and urea showed a higher increase than normal values, while the content in calcium and triglycerides was more reduced. For the month of June, the average values of the analyzed blood biochemical parameters that did not fall within the limits provided by the specialized literature and that showed a significant increase were: ALT-GPT -24.13 U/L, Gamma-GT - 37.87 U/L, and total proteins - 82.93 g/L. Unlike the previously mentioned months, in July, in addition to the increase in the biochemical parameters represented by ALT and Gamma, in the blood of the animals sampled in the study, a reduced content of urea is added, symbolizing the fact that in July, the protein intake was not provided in the feed. For the month of August, following the determinations made on the serum samples, all the parameters were in the graph with the exception of two, thus a relatively higher content of alanine aminotransferase ALT-GPT was observed, the average value being 25.93 U/l, and for gamma -GT showed an average value of 26.67 U/L. Elevated alanine aminotransferase is an indicator of liver disease. Mild to moderate elevations of ALT (up to four to five times normal) may occur in nonhepatic conditions such as inflammatory gastrointestinal disease, heart failure, and hemolytic anemia (Stoev, 2015). Increased gamma-glutamyltransferase - indicates diseases with liver localization. GGT is an enzyme that is found throughout the body but is mostly found in the liver. When the liver is damaged, GGT can leak into the blood. High levels of GGT in the blood can be a sign of liver disease or damage to the bile ducts. The increase in urea indicates an alteration of liver function, but also other changes, such as dehydration, heart disease, shock, and urethral obstructions (Streit et al., 2012). In several studies, diets contaminated with FUM led to an increase in liver enzymes, such as gamma-glutamyl transpeptidase (GGT). Blood GGT levels can be used to detect chronic, subacute, or acute liver disease and an increase in these two liver enzymes can be associated with fatty liver syndrome in ruminants. Increases in these liver enzymes were detected in dairy cows receiving a combination of 994 ppb FUM and 733 ppb DON (Gallo et al., 2020), as well as beef cattle receiving 3.5 ppm FUM and 1.7 ppm DON in combination (Duringer et al., 2020). Elevated levels of these liver enzymes are a sign of liver damage caused by ingestion of FUM. Regarding the biochemical analyses, Table 6 presents the statistical interpretation of the values of biochemical parameters of the blood in the serum.

Table 6. Statistical interpretation of the values of blood biochemical parameters in the serum

Parameter		Month						ANOVA	LSD
		March (3)	April (4)	May (5)	June (6)	July (7)	August (8)		
Milk production (L)	X±sD	31.02±2.93	31.22±2.34	28.11±1.49	28.03±1.06	16.13±3.90	16.09±3.40	$p = 0.000$ (**$p < 0.001$ *****)	3:5,3:6,3:7,3:8 4:5,4:6,4:7,4:8 5:7,5:8,6:7,6:8
	Min.	26.33	27.88	25.72	26.54	10.88	10.01		
	Max	35.50	35.98	30.63	29.99	22.16	20.98		
Albumin (g/L)	X±sD	34.87±3.20	33.87±3.23	29.60±5.90	34.27±5.54	33.47±3.66	34.13±5.38	$p = 0.035$ (**$p < 0.05$ ***)	3:5,4:5, 5:6,5:7, 5:8
	Min.	26.00	28.00	15.00	27.00	27.00	18.00		
	Max	40.00	41.00	40.00	48.00	40.00	38.00		
ALP-DEA (U/L)	X±sD	87.33±17.51	93.33±19.76	87.60±20.22	74.67±42.22	120.47±18.15	122.87±49.58	$p = 0.000$ (**$p < 0.001$ *****)	3:7,3:8,4:7,4:8 5:7,5:8,6:7,6:8
	Min.	62.00	59.00	46.00	30.00	104.00	68.00		
	Max	111.00	134.00	127.00	176.00	167.00	255.00		
ALT-GPT (U/L)	X±sD	24.80±5.60	24.67±4.37	23.07±6.62	24.13±5.33	27.27±4.35	25.93±6.95	$p = 0.417$ (**$p > 0.05$ ns**)	5:7
	Min.	16.00	15.00	6.00	18.00	15.00	12.00		
	Max	37.00	33.00	35.00	36.00	35.00	36.00		
AST-GPT (U/L)	X±sD	102.27± 35.20	104.93± 34.01	95.60± 22.25	109.80± 45.29	84.73± 27.87	78.13± 15.67	$p = 0.054$ (**$p > 0.05$ ns**)	3:8,4:8,6:7,6:8
	Min.	58.00	64.00	48.00	50.00	47.00	54.00		
	Max	190.00	171.00	142.00	200.00	136.00	116.00		
Total bilirubin (mg/dL)	X±sD	0.08±0.08	0.09±0.09	0.05±0.06	0.08±0.04	0.23±0.45	0.08±0.14	$p = 0.199$ (**$p > 0.05$ ns**)	5:7,6:7,7:8
	Min.	0.01	0.01	0.01	0.01	0.01	0.01		
	Max	0.26	0.33	0.20	0.19	1.64	0.54		
Calcium (mg/dL)	X±sD	8.46±0.60	8.46±0.63	7.48±0.94	8.39 ±1.04	8.54±0.47	8.34±0.55	$p = 0.001$ (**$p < 0.001$ *****)	3:5,4:5,5:6,5:7 5:8
	Min.	7.40	6.69	5.46	7.15	7.41	6.61		
	Max	9.68	9.41	9.00	11.75	9.26	8.88		
Cholesterol (mg/dL)	X±sD	201.73±55.77	216.53±60.97	180.87±59.29	192.27±41.43	196.33±33.65	175.13±37.86	$p = 0.248$ (**$p > 0.05$ ns**)	4:8
	Min.	99.00	89.00	41.00	110.00	124.00	129.00		
	Max	292.00	351.00	302.00	273.00	242.00	242.00		
Creatinine (mg/dL)	X±sD	0.71±0.11	0.68±0.12	0.69±0.15	0.75 ±0.15	0.56±0.15	0.57±0.11	$p = 0.000$ (**$p < 0.001$ *****)	3:7,3:8,4:7,4:8 5:7,5:8,6:7,6:8
	Min.	0.51	0.53	0.50	0.56	0.17	0.38		
	Max	0.90	0.94	1.04	1.12	0.83	0.74		
Gamma-GT (U/L)	X±sD	34.20±18.66	37.80±24.23	25.00±11.28	37.87±15.28	25.20±10.22	26.67±7.14	$p = 0.045$ (**$p < 0.05$ ***)	4:5,4:7,5:6,6:7
	Min.	13.00	6.00	6.00	15.00	7.00	17.00		
	Max	83.00	93.00	53.00	73.00	41.00	39.00		
Total protein (mg/dL)	X±sD	71.67±6.79	74.80±6.16	67.80±8.17	82.93±9.49	75.33±6.78	79.27±3.69	$p = 0.000$ (**$p < 0.001$ *****)	3:6,3:8,4:5,4:6 , 5:6,5:7,5:8,6:7
	Min.	60.00	60.00	49.00	75.00	65.00	73.00		
	Max	82.00	86.00	83.00	107.00	92.00	85.00		
Triglycerides (mg/dL)	X±sD	6.94±3.40	5.14±3.81	2.80±2.88	14.47 ±3.72	18.27±4.32	16.67±6.31	$p = 0.000$ (**$p < 0.001$ *****)	3:5,3:6,3:73:8, 4:6,4:74:8,5:6, 5:7,5:8,6:7
	Min.	0.10	0.01	0.00	10.00	12.00	5.00		
	Max	12.00	11.00	8.00	24.00	28.00	30.00		
Ureea (mg/dL)	X±sD	35.80±6.61	34.00±5.36	27.53±5.00	22.53±8.95	8.87±5.53	12.40±6.23	$p = 0.000$ (**$p < 0.001$ *****)	3:5,3:6,3:7,3:8 ,4:5,4:6,4:7,4: 8,5:6,5:7,5:8,6 :7,6:8
	Min.	21.00	21.00	14.00	11.00	1.00	0.00		
	Max	48.00	42.00	35.00	49.00	19.00	22.00		

p> 0.05 = insignificant / ns, p< 0.05 = significant / *, p< 0.01 = distinctly significant / **, p< 0.001 = highly significant / ***
ANOVA - Analysis of variance, LSD - Least Significant Difference

Milk production was significantly higher in the months of March, and April compared to the other months (p<0.001), while the average values of milk production were significantly lower in the months of July and August. Through the ANOVA test, it is found that there are very significant differences in milk production over the six months, and through the LSD test we note what these are, thus the months of March, and April show very significant differences (p<0.001) with the months of May, June, July, August, while the months of May, June show very significant differences ($p<0.001$) with the months of July and August. As the temperature is higher in the summer months, and as the body temperature also increases, cattle reduce their feed intake to alleviate heat stress, thus leading to a gradual decrease in milk production and a change in milk fat content milk (Jiangjing et al., 2019).

The albumin content was significantly higher in March, June, and August compared to the other months ($p<0.05$), while the mean values were significantly lower in April, May, and July. The lowest value recorded was 15.00 g/L, while the highest value was 48.00 g/L (Table 6). Between the albumin content during the six months, it is found that there are significant differences ($p<0.05$), according to the ANOVA and LSD statistical test.

Alkaline phosphatase content (ALP-DEA) was highly significant in July and August and less so in the other months ($p<0.001$). The lowest value recorded for all months was 30.00 U/L, while the highest value was 255.00 U/L (Table 6). The differences are highly significant ($p<0.001$), according to the ANOVA statistical test coupled with the LSD test.

The content in alanine aminotransferase (ALT-GPT) was significantly higher in July compared to the other months, but we are talking about a statistically insignificant correlation ($p>0.05$). Between the content of alanine aminotransferase during the six months, it is found that there is only one insignificant difference ($p>0.05$) and this is between May and July, according to the ANOVA statistical test coupled with the LSD test (Table 6).

It was found that the content in aspartate aminotransferase (AST-GOT) was found insignificant during the six months ($p<0.05$), according to the ANOVA test. The lowest value recorded for all months was 47.00 U/L, while the highest value was 200.00 U/L (Table 6). The LSD test highlights that there were insignificant differences ($p>0.05$) (Table 6). Regarding the dynamics of total bilirubin during the experimental period March-August, the correlations are insignificant (*$p>0.05$*) according to the ANOVA statistical test coupled with the LSD test, there being differences between the months of May and July, June and July, and July and August. The lowest value recorded for total bilirubin was 0.01 mg/dL, while the highest value was 1.64 mg/dL for all months (Table 6).

Regarding the calcium content, very significant differences ($p<0.001$) are found during the six months, according to the ANOVA statistical test. The LSD test highlights what these differences are, thus we note that between March and May, April and May, May and June, July and August there are very significant differences ($p<0.001$).

Correlations of cholesterol content are insignificant ($p>0.05$) according to the ANOVA statistical test. According to the LSD test, there were differences only between April and August. The lowest value recorded for cholesterol content was 41.00 mg/dL, while the highest value was 351.00 mg/dL for all months (Table 6). Regarding the creatinine content, the correlations are highly significant ($p<0.001$) according to the ANOVA statistical test coupled with the LSD test, there are differences between March and July and August, April, and July and August, May, and July and August and June with July and August (Table 6).

Glutamyl transpeptidase (Gamma-GT) was significantly higher in April, and June compared to the other months ($p<0.05$), while the lowest values significantly recorded were in May, July, and August. During the six months, it was found that the differences were significant ($p<0.05$), according to the ANOVA and LSD statistical test.

The total protein content was significantly higher in June compared to the other months ($p<0.001$). The correlations are highly significant ($p<0.001$) according to the ANOVA statistical test coupled with the LSD test, there are differences between March and June and August, April and May and June, May and June, July and August, but also between June and July (Table 6).

Triglyceride content was highly significant in July and August and less so in the other months ($p<0.001$), with differences between March and May, June, July, August, April, and May with June, July, August and between June and July ($p<0.001$) (Table 6).

According to the ANOVA test, coupled with the LSD test, urea had a higher content in March and April compared to the other months, the differences being highly significant ($p<0.001$).

To highlight the differences in the parameters during the studied period, it is observed that milk production, alkaline phosphatase, calcium content, creatinine, total proteins, triglycerides, and urea showed very significant differences during the 6 months, where $p<0.001$. Significant differences, where $p<0.05$ were found only in 2 of the 13 parameters, such as albumin and glutamyl transpeptidase, and non-significant differences were for ALT, AST, bilirubin, and cholesterol. The overview of the correlations of the analyzed biochemical parameters on bovine serum is presented in Table 7.

Table 7. Pearson correlation on the analyzed biochemical parameters of bovine serum

Pearson Corelation	a)	b)	c)	d)	e)	f)	g)	h)	i)	j)	k)	l)	m)
Milk production[a)] (L)	1	.002	-.419**	-.178	.290**	-.224*	-.091	.194	.389**	.214*	-.214*	-.665**	.740**
Albumin[b)] (g/L)	0.002	1	.179	.631**	.278**	-.043	.760**	.391**	.412**	.079	.252*	.254*	.293**
ALP-DEA[c)] (U/L)	-0.419**	0.179	1	.220*	-.065	.190	.306**	-.019	-.103	.006	-.008	.266*	-.298**
ALT-GPT[d)] (U/L)	-0.178	0.631**	0.22*	1	.368**	-.201	.437**	.570**	.080	.047	.133	.253*	.200
AST-GOT[e)] (U/L)	0.290**	0.278**	-0.065	0.368**	1	-.106	.228*	.313**	.301**	.627**	.134	-.235*	.377**
Total bilirubin[f)] (mg/dL)	-0.224*	-0.043	0.190	-0.201	-0.106	1	.087	-.167	.034	.062	.109	.182	-.194
Calcium[g)] (mg/dL)	-0.091	0.760**	0.306**	0.437**	0.228*	0.087	1	.292**	.401**	.109	.521**	.338**	.148
Cholesterol[h)] (mg/dL)	0.194	0.391**	-0.019	0.570**	0.313**	-0.167	0.292**	1	-.034	.166	.110	-.070	.338**
Creatinine[i)] (mg/dL)	0.389**	0.412**	-0.103	0.080	0.301*	0.034	0.401**	-0.034	1	.121	.184	-.142	.440**
Gamma-GT[j)] (U/L)	0.214*	0.079	0.006	0.047	0.627**	0.062	0.109	0.166	0.121	1	.195	-.114	.202
Total protein[k)] (g/L)	-0.214*	0.252*	-0.008	0.133	0.134	0.109	0.521**	0.110	0.184	0.195	1	.390**	-.143
Triglycerides[l)] (mg/dL)	-0.665**	0.254*	0.266*	0.253*	-0.235*	0.182	0.338**	-0.070	-0.142	-0.114	0.390**	1	-.553**
Urea[m)] (mg/dL)	0.740**	0.293**	-0.298**	0.200	0.377**	-0.194	0.148	0.338**	0.440	0.202	-0.143	-0.553**	1

* - statistically significant correlation ($p<0.05$); **- distinctly significant correlation ($p<0.01$).

The Pearson correlation test was used to evaluate the relationships between biochemical parameters during the 6 months, thus it is observed that milk production was significantly positively correlated with AST, creatinine, and urea and negatively correlated with ALP and triglycerides ($p<0.01$). Albumin was significantly positively correlated with ALT, AST, calcium, cholesterol, creatinine, and urea. ALP was significantly positively correlated with calcium and negatively with milk and urea production. ALT was significantly positively correlated with albumin, AST, calcium, and cholesterol, and AST was significantly positively correlated with milk production, albumin, ALT, cholesterol, creatinine, gamma, and urea. Calcium was significantly positively correlated with albumin, ALP, ALT, cholesterol, creatinine, total proteins, and triglycerides. Cholesterol was significantly positively correlated with albumin, ALT, AST, calcium, and urea. Creatinine was significantly positively correlated with milk production, albumin, calcium, and urea. Gamma GT was significantly positively correlated only with AST. Total proteins were significantly positively correlated only with calcium and triglycerides. Triglycerides were significantly positively correlated only with calcium and total protein and negatively correlated with milk production and urea. Also, urea was significantly positively correlated with milk production, albumin, AST, and cholesterol and significantly negatively correlated with ALP and triglycerides. The other correlations are insignificant. We observe that positive correlations are distinguished for most blood biochemical parameters and only a few negative ones such as milk production, ALP alkaline phosphatase, triglyceride, and urea content. The evaluation of urea, triglycerides, cholesterol, creatinine, and other parameters provides an opportunity to expect a healthy production in animals, this evaluation bearing the name of blood profile test. Unlike other authors, where ALT usually presents a low level, in the present article, from the results of biochemical analyses on bovine serum, it emerged that the concentrations of ALT, gamma-Gt, and urea were higher than the rest of the parameters. Blood concentrations of enzymes including AST, GGT, and ALT are considered indicators of liver function and are also correlated with metabolic diseases such as ketosis (Li et al., 2016). As in Joo S.'s article, triglycerides showed a lower value. The reduction of triglycerides can lead to the accumulation of triglycerides from the blood in the liver, and this phenomenon can induce metabolic diseases (Joo, 2021).

CONCLUSIONS

During the experimental period, from a mycotoxicological point of view, the results of the feed samples were in accordance with the specialized literature and fell within the maximum limits allowed by the standard (Regulation (EC) no. 1881/2006).

The only biochemical parameters that did not fall within the limits of the reference range and that showed a relative increase were represented by ALT-alanine aminotransferase and Gamma Gt-glutamyl aminotransferase. An above-normal content was also observed in the case of urea. Parameters that had a value lower than the accepted limit were represented by triglycerides. All cows involved in the study were apparently healthy at the time of sample collection, but the resulting biochemical analyses may suggest mild liver disorders, according to the literature. Regarding the increase above the normal limit of ALT, Gamma-Gt, and urea parameters, it should be mentioned that the differences were quite small. Likewise in the case of triglycerides. Reduced content of triglycerides showed that the studied animals were in a negative energy balance at that time.

The content of mycotoxins present in the feed, even if it was relatively low and within the limits stipulated by the standard, had no negative effects on the health of the animals studied, except in a very small percentage, the biochemical analyses show.

Consequently, certain scientific evidence regarding the adverse effects of mycotoxin ingestion on cattle health and performance is scarce and remains to be proven.

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THE INFLUENCE OF THE SIZE OF THE PIG FARM ON THE PRODUCTIVITY OF PIGLETS AND THE EFFICIENCY OF THEIR BREEDING

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Abstract

The article investigated the productivity of Danish-bred piglets during rearing in small and medium-sized pig farms and large industrial complexes, as well as the effectiveness and components of rearing costs and the influence of pig farm size on them. In small pig farms, it was found that piglet survival rate during rearing was 0.24-0.45% higher and growth intensity was 3.15-5.46% higher during this period. The absolute gains were 0.59-2.50% higher and the weight of piglets at transition to fattening was 4.53-8.50% higher than in medium and large size farms, respectively. At the same time, animals in large farms had 4.15% lower birth weight, 3.17% absolute and 2.19% average daily gains and 1.60% lower weight of animals at transition to fattening compared to medium size farms. It has been shown that the most effective feed was used by animals reared in medium capacity farms. The feed cost per piglet was lowest for animals reared on medium-sized farms, 9.67% lower than for animals reared on small farms during this period, and 9.70% lower than for animals reared on large pig farms.

Key words: *cost price, feed conversion, growth, piglets, rearing.*

INTRODUCTION

Pig farming plays a critical role in global food production (Ruckli et al., 2022), and pigs are a valuable source of meat and provide a significant portion of global pork production (Rauw et al., 2020). At the same time, the efficiency of production depends on many factors, such as the age and initial weight of pigs at the time of placing them for rearing (Shvachka, 2023), the duration of the rearing period (Nechmilov, 2019), housing conditions during this period, the size of the group of piglets in the machine, floor space, and the feeding front for one piglet and another.

The livestock sector is rapidly industrialized, especially in developing countries (Mirle, 2012). According to reports (Povod, 2021), pig farms in Ukraine are usually divided into small farms with up to 6,000 animals, medium farms with 6,000 to 15,000 animals, and large pig complexes with a capacity of more than 15,000 animals. Today, there are 1,700 pig complexes operating in Ukraine, in which there are 3.7 million pigs, and only 95 enterprises have more than 5,000 pigs (Myhalko, 2021). According to the known data (Dudin et al., 2013; Voloshchuk et al., 2014), large industrial enterprises use the latest achievements of science and technology, which allow more rational use of space and material resources, and carry out narrow specialization of production processes with the involvement of narrowly qualified specialists who have more important skills in the competitive process The concentration and specialization of pork production, which was accompanied by the introduction of industrial technology for pig farming, contributed to a significant reduction in labor costs for pig maintenance due to the automation of production processes (Povod &

Shpetny, 2018; Tsereniuk, 2017). Of the two possible directions of pig farming development: extensive and intensive - intensive is the most important. Intensification of pig farming is a development path in which, due to the increase in the cost per animal and a more complete and rational use of the means of production, an increase in the productivity of the animals and, consequently, an increase in pork sales is achieved and, therefore, an increase in pork production (Tsereniuk, 2017). Intensive pig production offers many advantages in terms of animal health, food safety, hygiene and biosecurity as well as some welfare aspects compared to extensive production (Maes et al., 2019). According to reports (Povod, 2022), the industrialisation of pork production on small farms increased the production of products per 1 m2 of production area by 2.22 times and the production of pork per worker by 1.44 times, while in medium-sized farms this production per 1 m^2 of production area increased by 2.01 times and in large farms by 3.17 times, while the gross production of products per worker increased by 3.54 times in medium-sized farms and by 4.61 times in large industrial complexes. There are different opinions about the welfare and productivity of piglets when they are kept in pig farms with different capacities. Thus, according to data (Nechmilov, 2019; Windhorst, 1998), it is known about the improvement of productive indicators in piglets due to the use of industrial technology at large industrial complexes due to the creation of more comfortable conditions for their keeping.

At the same time, there are a number of publications (Shpetny & Povod, 2016; Shpetny & Povod, 2018; Shpetny, 2019) claiming that large group housing of piglets is usually used in industrial complexes where staff attention to the animals decreases and their safety and productivity deteriorates. While other authors point out that piglet productivity does not depend on the capacity of the farm and focus on the technological aspects of husbandry, including the intensity of pig growth (Lebret, 2008). It is known that pig growth depends on their genetic affiliation (Šprysl et al., 2005), feeding systems (Rauw et al., 2017; Nechmilov, 2019), health (Cornelison et al., 2018), housing conditions (Povod & Shpetny, 2016; Lykhach, 2015) and production process management methods (da Fonseca de Oliveira et al., 2023).

Thus, farm size can influence pig growth and feed consumption through various factors related to management, resources and infrastructure. Larger farms often use more specialized management methods and trained personnel (Oosthuizen & Janovsky, 1981) to deal with specific tasks, such as nutritionists, veterinarians, and farm managers. This can lead to more efficient and effective management strategies, including optimized feeding programs and better monitoring of pig rearing. Consequently, well-managed large farms can achieve high growth rates and feed efficiency compared to farms with medium and small pig herds (Izmaylov et al., 2022; Makara et al., 2019).

In addition, large farms usually have more space to raise pigs, and sufficient space allows pigs to move freely, increase their physical activity, and behave more naturally. When pigs have sufficient space, they are less likely to suffer crowding and compete for resources, which can cause stress in some herds and stunt growth (Chidgey, 2023; Nielsen et al., 2022). In addition, larger farms may be able to house pigs separately at different growth stages, which optimizes conditions and promotes growth. Another factor somewhat related to farm size and pig housing technology is group size (Spoolder et al., 1999). It can indirectly influence pig growth during the rearing period through social tensions in the herd. It has been proven that pigs are social animals that naturally form a social hierarchy (Signoret et al., 1975; Tong et al., 2019). However, contrary to what has been said, there is an opinion that individual pigs in small groups have more opportunities to establish their social status, keep it stable longer, and consequently reduce competition for resources such as food and water and minimize hierarchical reorganization of the herd. This can lead to higher growth rates because pigs are exposed to less stress and can access resources more easily and expend less energy and hierarchy struggles (Lykhach et al., 2020; Riedel et al., 2012; Rhim, 2012).

All of the above factors can have varying degrees of influence on small and medium-sized farms and large industrial pig breeding complexes. Therefore, taking into account the above information, the study of the influence of the size

of the pig enterprise and the initial weight of piglets when growing them is relevant and timely.
Our hypothesis is that increasing the number of pigs per farm and as a consequence of its size will increase the productivity of pigs.
The objective of our experiment was to determine the effect of farm size on pig productivity, feed conversion, and farm economic efficiency.

MATERIALS AND METHODS

The material for the study was the productive qualities and economic indicators during the rearing of hybrid pigs obtained from crossbred sows of the Danish Landrace and the Danish breed Large White, inseminated with semen from boars of the terminal lines of the Danish Duroc. The object of the study was the technological processes in 80 farms in the Kingdom of Denmark. Data from open sources of the rating analysis of DB-Tjek pig farms for 2021 in the Kingdom of Denmark, conducted by the consultancy Svine Rådgivningen, were used for the analysis. The data to be studied were randomly selected from this report. In order to investigate the influence of farm size, all pig farms studied were divided into three groups according to the extent of annual rearing of piglets.
The first group included small farms with an annual production of 6,000 to 15,000 piglets, the second group included medium-sized pig farms with an annual production of 15,001 to 30,000 piglets, and the third group included pig farms complexes with an annual production of more than 30,000 pigs. Based on the data in this report, the growth intensity of piglets and their preservation during rearing, daily consumption and consumption of feed per 1 kg of growth, costs of rearing piglets and their components were analyzed. A single-factor analysis of variance was used to determine the effect of farm size on the main productivity parameters of piglet rearing and the economic indicators of the farm.

RESULTS AND DISCUSSIONS

As shown in Table 1, the weight of piglets when placed on rearing depended on the size of the pig farm. At 7.6 kg, it was highest in small pig farms. As the size of the farm increased, the weight of piglets when they were put into the nursery decreased. Thus, it was lower by 0.32 kg (4.53%) in medium-sized farms and by 0.60 kg (8.5%) in large pig farms than in small farms ($p<0.05$). At the same time, the weight of piglets at birth in large farms was 0.28 kg or 4.15% lower than in medium farms. The greater mass of piglets at the beginning of rearing contributed to their higher growth energy. Thus, animals raised on small hog farms during this period were likely to have higher average daily gains by 15.9 g (3.35%) ($p<0.05$) compared to peers raised on medium farms and by 29.9 g (5.46%) ($p<0.01$) compared to analogues raised on large hog farms at this time. While the average daily gains of the animals of the third group turned out to be 10.0 g or 2.19% worse compared to the analogues of the second group.

Table 1. Growth rates of piglets depending on the size of the pig farm

Indicators	I Group (6000-15000 pigs per year)	II Group (15001-30000 pigs per year)	III Group (more than 30000 pigs per year)	p-value
Number of pig farms taken into account (n)	28	32	20	-
Weight of piglets at the beginning of rearing, kg	7.0 ± 0.29^{a}	6.7 ± 0.23^{ab}	6.4 ± 0.10^{b}	<0.05
Average daily increase, g	473.7 ± 5.92^{a}	457.9 ± 7.26^{ab}	447.9 ± 6.51^{b}	<0.01
Absolute growth, kg	25.2 ± 0.46^{a}	24.5 ± 0.37^{a}	25.3 ± 0.57^{a}	>0.05
Weight of piglets at the end of rearing period, kg	32.2 ± 0.43^{a}	31.3 ± 0.38^{a}	31.8 ± 0.50^{a}	>0.05
Preservation of piglets during rearing period, %	97.8 ± 0.16^{a}	97.6 ± 0.21^{a}	97.4 ± 0.26^{a}	>0.05

Note: Different lowercase letters (a, b) indicate statistical differences between the columns.

Taking into account the almost identical period of piglet rearing in all the complexes, the absolute growth of the piglets during this period was naturally higher in the animals of the first group, which, according to this indicator, exceeded the analogues of the second group by 0.63 kg (2.5%) and those of the third group by 0.15 kg (0.59%). The piglets of the third group had the highest indicators of absolute growth, exceeding the analogues of the second group by 0.78 kg (3.17%) and the analogues of the first group by 0.15 kg (0.59%) by this indicator. Taking into account the higher initial mass at the time of rearing and the greater absolute growth during rearing, the weight of the piglets at the end of rearing was naturally higher on small pig farms. Thus, this indicator was 32.28 kg for the animals in the second group, 0.95 kg (2.94%) for the animals in the third group and 0.45 kg (1.35%) for the animals in the third group compared to the control group. At the end of rearing, the piglets of the third group were 0.50 kg (1.60%) heavier compared to the peers of the second group.

It was also found that small pig farms had a slightly better survival rate of piglets during the growth period compared to medium and large farms.

Thus, small farms were found to have 0.24-0.45% better survival rate of piglets during rearing and 3.15-5.46% higher growth intensity during this period, 0.59-2.50% higher absolute gains and 4.53-8.50% higher weight of piglets at transition to fattening compared to medium and large farms, respectively.

At the same time, animals from large farms had 4.15% lower birth weight of piglets, 3.17% higher absolute growth, 2.19% higher average daily growth and 1.60% lower weight of animals at the transition to fattening compared to medium farms.

When analyzing the efficiency of feed use (Table 2), it was established that piglets raised on small pig farms consumed about 0.04 kg (4.44%) more feed than their counterparts raised during this period on medium-sized farms and by 0.06 kg (7.78%) more than their peers who grew up on large pig farms. In turn, the latter animals consumed 0.03 kg (3.49%) more feed compared to animals that were raised on medium-sized farms at that time.

Table 2. Efficiency of use of fodder depending on the size of the pig farm

Indicators	I Group (6000-15000 pigs per year)	II Group (15001-30000 pigs per year)	III Group (more than 30000 pigs per year)	p-value
Number of pig farms taken into account (n)	28	32	20	-
Average daily feed consumption, kg	0.8±0.014[a]	0.7±0.010[a]	0.7±0.011[a]	>0.05
Feed consumption per 1 kg of growth, kg	1.7±0.02[a]	1.6±0.02[a]	1.6±0.03[a]	>0.05
Feed consumption per head, kg	43.5±0.92[a]	41.8±0.81[a]	42.9±1.36[a]	>0.05
Feed consumption per head, DKK	114.1±2.50[a]	103.1±1.8[b]	113.1±3.22[a]	<0.001
Feed consumption per 1 kg of growth, DKK	4.5±0.073[a]	4.2±0.050[b]	4.4±0.101[a]	<0.001

Note: Different lowercase letters (a, b) indicate statistical differences between the columns; DKK - Danish krone

Higher average daily feed intake, despite higher growth energy, resulted in poorer feed conversion in the animals of the first group. Thus, it was 0.03 kg (1.59%) higher than in the animals of the second and third groups. Taking into account the different absolute growth of animals in complexes with different capacity, feed consumption per head was highest in piglets of the first group and lowest in animals of the second group. The difference was 1.71 kg or 3.93%. Piglets raised in large industrial complexes consumed 1.08 kg (2.59%) more feed per head compared to their counterparts raised in medium-sized farms at that time, and 0.63 kg (1.44%) less compared to their peers raised in small farms. Accordingly, the cost of feed to raise an animal was lowest for the piglets raised on medium-sized farms, based on the cost of one kilogram of feed. This figure was probably 11.04 Danish kroner (9.67%) lower than for low capacity farms and 10.00 Danish kroner (9.70%) lower than for large pig farms. The feed cost of raising a piglet on large pig farms was again only 1.04 Danish kroner, or 0.91%, compared to animals on small pig farms.

Feed costs for achieving one kilogram of growth were lowest for animals in the second group. Thus, they were 0.33 Danish kroner (7.28%) lower than this indicator in the animals of the first group and 0.26 Danish kroner (6.19%) lower than in the animals of the third group.

In this way, the feed was used most effectively by animals in farms with medium capacity. They have a 3.93% lower amount of feed per head compared to the animals raised in small pig farms at that time and 2.59% lower compared to the animals raised in large pig farms at that time. Feed cost per piglet was lowest for animals from medium-sized farms, 9.67% lower than for animals from small farms, and 9.70% lower than for animals from large pig farms. Mean cost per 1 kg of piglet gain was lowest in medium capacity farms, i.e. 6.19% better than the same indicator in large farms and 7.28% better than in small farms.

The most important indicator of pork production is its economic efficiency. Table 3 shows the costs of raising piglets and their main components in farms with different capacities. As can be seen from this table, the cost of piglets used for rearing is highest in small farms due to their higher live weight. In contrast, the price of a pig taken out of rearing was already highest in large farms and lowest in medium farms. This also caused a different cost of raising one head of piglets. Thus, the lowest cost per piglet was found on small pig farms, and it increased with the growth of the farm's capacity. Thus, on medium-sized pig farms, it turned out to be 4.70 Danish kroners (2.71%) higher compared to small ones. At the same time, the cost of rearing in large pig farms was 4.71 Danish kroners (2.45%) higher compared to this indicator in medium-sized farms and 9.40 Danish kroners (5.03%) in comparison with small pig farms.

Table 3. Efficiency of growing piglets depending on the size of the pig farm

Indicators	**I Group (6000-15000 pigs per year)**	**II Group (15001-30000 pigs per year)**	**III Group (more than 30000 pigs per year)**	**p-value**
Number of pig farms taken into account (n)	28	32	20	-
The cost of 1 head at the beginning of breeding, DKK	264.1±4.20^{a}	259.1±3.81^{a}	260.0±2.73^{a}	>0.05
Cost of 1 head when transferred for fattening, DKK	451.1±3.66^{a}	450.8±2.94^{a}	456.5±2.01^{a}	>0.05
The cost of raising one head, DKK	187.0±4.46^{a}	191.7±3.66^{a}	196.4±2.68^{a}	>0.05
The share of feed in the cost of raising 1 head, %	61.7±1.62^{a}	54.0±0.89^{b}	57.7±1.80^{a}	<0.001
Part of veterinary costs for breeding 1 head, DKK	12.2±1.79^{a}	11.9±1.51^{a}	13.67±1.53^{a}	>0.05

Note: Different lowercase letters (a, b) indicate statistical differences between the columns; DKK - Danish krone

The cost structure for rearing pigs on farms with different capacities proved to be different. However, feed accounted for the largest share of rearing costs. For example, on small pig farms, feed accounted for 61.7% of the cost structure for rearing a piglet, while on large pig farms and medium-sized farms, the share of feed in the cost of rearing a piglet was 4.0 and 3.69% lower, respectively, compared to small farms. We also examined the share of medicines and veterinary services in the total costs of rearing piglets. Medium-sized farms also scored the best on this indicator, as the share of veterinary costs was 0.31 and 1.75% lower, respectively, compared to small farms and large pig complexes.

Thus, the cost of rearing a piglet was lowest on small farms, 2.51% lower than on medium farms and 5.03% lower than on large complexes.

The share of feed in the cost of rearing piglets was 7.64% lower in medium capacity farms than in small farms and 3.69% lower than in large pig farms. The percentage of medicines, veterinary drugs and services of a veterinarian was 1.44% in large pig farms compared to small farms and 1.75% compared to medium capacity farms.

Using a factorial analysis of variance, a probable influence of 8.2% of farm capacity on piglet growth intensity during rearing was found (Figure 1).

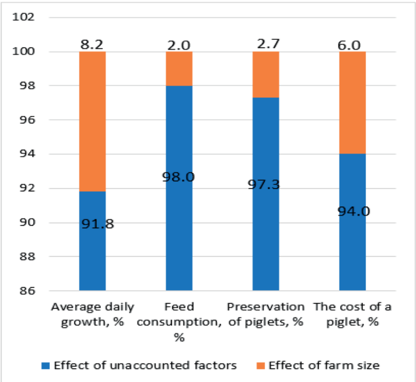

Figure 1. The influence of the size of the pig farm on average daily gains, survival of piglets, feed conversion and the cost of raising one piglet

In contrast, the influence on conservation and feed consumption per 1 kg of growth was significantly lower and unlikely at 2.7 and 2.0%, respectively.

Also unlikely was the influence of the capacity of the pig complex on the cost of rearing a piglet at a value of 6.0%. Thus, the capacity of the pig complex had a probable influence of 8.2% on average daily gains and an improbable influence on the cost price of piglets, their conservation and feed conversion (Table 4).

Table 4. Results of one-factor variance analysis

Indicators	SS	MS	F	F crit (α = 0.05)	P-value	HIP 0.05
Average daily growth						
Total, Cy	101442.99					
Size of the pig farm, A	8300.60	4150	3.39	3.12	0.03901	19.215
Unaccounted for factors, Cz	93142.39	1226				
Feed consumption						
Total, Cy	1.00					
Size of the pig farm, A	0.02	0.0101	0.78	3.12	0.45982	0.062
Unaccounted for factors, Cz	0.98	0.0129				
Preservation of piglets						
Total, Cy	88.38					
Size of the pig farm, A	2.39	1.2	1.05	3.12	0.35349	0.584
Unaccounted for factors, Cz	86.00	1.1				
The cost of a piglet						
Total, Cy	32141.95					
Size of the pig farm, A	1934.45	967	2.47	3.12	0.09165	10.801
Unaccounted for factors, Cz	30207.50	392				

Comparing the results of our research with the reports (Nechmilov, 2019; Windhorst, 1998; Izmaylov et al., 2022; Makara et al., 2019) indicating improvement of productive indicators in piglets due to the use of industrial technology in large industrial complexes, we note a deterioration of average daily gains in piglets kept in large farms compared to medium and small farms. Moreover, our results did not agree with the other conclusions (Tsereniuk, 2017), which spoke of an improvement in the productivity of pigs when the size of the farm was increased and, accordingly, the number of pigs kept there was increased. We found an increase in productivity in medium-sized farms and a decrease in productivity in farms with the largest number of livestock, which is consistent with the results (Shpetny & Povod, 2016; Shpetny & Povod, 2018; Shpetny 2019), but contradicts the data (Lebret, 2008), which found the absence of a significant effect of farm size on the productive characteristics of pigs. However, there were reports (Shpetny, 2019) where the author focused on the deterioration of the conservation index with the increase in the size of the farm and the number of pigs on it, which we could not confirm in our experiment.

Moreover, our results of the analysis of feed costs in farms of different sizes do not agree with the data (Izmaylov et al., 2022; Makara et al., 2019) reporting that large farms can have higher feed efficiency than farms with medium and small livestock. Swine On the contrary, we found that feeds are used more efficiently on medium-sized farms than on large and small farms and on swine farms.

CONCLUSIONS

It was found that in small pig farms, piglets are better maintained during rearing, their growth intensity during this period is higher, absolute gains are greater, and piglets have a higher weight at the transition to fattening than in medium and large farms.

Feed was used most effectively by animals in medium capacity farms. They have the lowest amount of feed per animal and the lowest feed cost, both per piglet and per 1 kg gain.

The cost of raising one piglet was the lowest on small farms, while its fodder and veterinary costs were on farms of medium capacity.

The size of the pig complex had a probable effect of 8.2% on average daily gains and did not affect the cost price of piglets, their preservation and feed conversion.

Our hypothesis of increasing pig productivity with increasing farm size was only partially true for medium-sized pig farms, but not for large-sized ones.

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EPIDEMIOLOGY, DIAGNOSIS, TREATMENT, CONTROL AND ECONOMIC IMPACT OF BRUCELLOSIS

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Abstract

Brucellosis is an infectious disease produced by bacteria of the genus Brucella spp. This disease mainly affects cows, sheep and goats, pigs and dogs. It is a zoonosis in humans it can be transmitted by 4 species of Brucella: B. suis, B. abortus, B. canis and B. melitensis, the latter being the most virulent. In animals the disease has a higher frequency of transmission during birth or abortion this being possible due to the fact that the bacteria colonize in the pregnant uterus. Once in the external environment, bacteria can survive for a long time, especially in wet and cold conditions, thus being able to be ingested by other animals. Colonization of bacteria at the level of the udder causes milk contamination. Male sperm, blood, urine and faeces of sick animals are also sources of contamination. The disease is transmitted to humans through the consumption of unpasteurized or insufficiently pasteurized milk or dairy products, consumption of meat prepared thermally insufficient or by direct contact with the infected animals, bacteria that enter the body through wounds on the skin or mucous membranes. The people most exposed to infection with Brucella spp. are represented by veterinarians, workers in slaughterhouses or farms, microbiologists. Human-to-human transmission of this zoonosis has proven to be extremely rare, but nursing mothers can transmit the infection to their children.

Key words: *Brucellosis, Burcella spp., humans, zoonosis.*

INTRODUCTION

Bacteria of the genus *Brucella* are small, coccoid or colibacillary bacteria, non-encapsulated and non-sporulated, facultative intracellular, Gram-negative. Regarding cultivation conditions and cultural characteristics, sponges need special environmental conditions to develop. The culture media they prefer are broth, agaul and potato, to which one or more of the following ingredients are added: glycerin 2%, glucose 1% or serum 1-5%. Cultural aspects are different depending on the environment. In the liquid environment, after a few days of incubation, moderate turbidity is observed, gray deposit, sometimes a ring is visible on the surface. On a solid medium, the cultural aspects differ depending on the medium used. On glycero-glucose agar, the colonies are small, round, opaque and glossy. On glycerinated potato medium, the colonies of *Brucella* appear glossy, yellowish in color, with aging they become chocolate-brown. In cultures, the bacteria last up to about 2 months. Brucellosis, also called Malta fever or wavy fever, is an anthropozoonosis described for the first time in Malta, in 1861. It is found globally and present in most countries, the incidence being different depending on the number of inhabitants and receptive animals (Qureshi et al., 2023; Şuler et al., 2019).

In Romania, it was identified for the first time in cattle in 1923, followed by pigs in 1934 and rams in 1958. It was diagnosed in humans for the first time in 1939, the 4 transmissible *Burcella* species being *B. suis, B. abortus, B. canis* and *B. melitensis*, the latter being the most widespread worldwide (Zamri-Saad & Kamarudin, 2016; Díaz Aparicio, 2013).

In animals, the symptoms are different. In cattle, the main symptom is the abortion that occurs in the 6-7 months of gestation, the fetal fluids are cloudy, yellowish in color, placental retention, endometritis, the decrease in volume and the change in the composition of the milk. In males, epididymitis, testicular enlargement, fever are observed. The incubation period can last between 10 and 239 days, depending on the physiological state of the infected organism (eur-lex.europa.eu; Hull & Schumaker, 2018).

In sheep and goats abortion occurs in the 3-4 months of gestation, placental retention with temporary sterility, mammary glands and nodules in the mammary gland, changes in milk - milk with blood clots and in males epididymitis and reduced fertility (Qureshi et al., 2023; Hull & Schumaker, 2018).

Abortion does not occur in sows, farrowings have time to term, but the viability of the products is reduced, placental retentions and mammitis have a lower frequency, but uterine infections, inflammation of the lymph nodes and arthritis occur instead. In males, inflammation of the testicles can be observed (Qureshi et al., 2023).

In humans, the symptoms of brucellosis are varied, fever being a characteristic symptom. This may be persistent or intermittent, accompanied or not by night sweats. Excessive fatigue and general weakness, muscle and joint pains and swelling that can affect mobility, headache that can vary from mild to severe, constant or intermittent, gastrointestinal symptoms - nausea, vomiting, abdominal pain or diarrhea, and neurological - in more severe forms, severe headaches, confusion, sleep disorders or even depression may appear, they are manifestations that are part of the symptomatology of brucellosis in humans. The average duration of the incubation period is 2 weeks, but it can last between 5 days and several months. *Brucella* are transported in the lymphatic system and can replicate there locally; they can also replicate in other organs such as the liver, spleen, kidneys, breast tissue or joints, causing both localized and systemic infections (Zamri-Saad & Kamarudin, 2016).

MATERIALS AND METHODS

This article was made through a thorough analysis of bibliographic sources from the specialized literature, relevant to the topic addressed, emphasizing the information related to epidemiology, diagnosis, treatment, control and the economic impact of brucellosis.

RESULTS AND DISCUSSIONS

As far as the epidemiology of the disease in animals is concerned, it is predominantly enzootic, the transmission of the infection from one animal to another being carried out mainly directly, through sexual intercourse or indirectly, through water, feed, various species of ticks or other insects hematophagies. The high frequency of spreading in the external environment occurs during parturition or abortion, considering the tropism of *Brucella* for the pregnant uterus. *Brucella* multiply only in the infected organism, having a long resistance in the external environment, which represents a danger for other animals, these being eliminated through the placenta, fetal fluids, abortions, uterine secretions, milk, because *Brucella* also colonizes the udder, urine and the sperm of sick males (Díaz Aparicio, 2013).

Regarding the sensitivity to environmental factors, it was found that at temperatures of 100°C bacteria are destroyed instantly, at 60-65°C between 5 and 15 minutes, in snow or ice they last up to 4 months. In faeces, litter or feed they have a resistance of up to 5 months, in soil or dust up to 10 weeks, in water up to 125 days. It lasts almost 3 months in frozen meat, and 30 days in salted meat. *Brucella* are sensitive to disinfectants such as chloramine, lime chloride, hydrochloric acid, cresol (Díaz Aparicio, 2013).

In humans, the transmission of the disease can be done by several ways, namely (Khoshnood et al., 2022):

- Direct contact with infected animals;
- Direct contact with aborted fetuses, fluids or membranes resulting from abortion;
- Direct contact with infected food;
- Consumption of infected food such as raw, unpasteurized or insufficiently pasteurized milk, cheese or curd;
- Exposure to a contaminated environment.

People can become infected through damaged skin or mucous membranes or by inhaling contaminated particles.

The people most exposed to the risk of infection with the *Brucella* bacterium are those who work in the agricultural field, farm workers or animal breeders, veterinarians, workers in slaughter-houses or in laboratories dealing with the diagnosis of brucellosis. It is not transmitted

from human to human (Khoshnood et al., 2022; Głowacka, 2018).
The morbidity of brucellosis is higher in spring and summer, when calving or abortions take place, especially in sheep and goats (Zamri-Saad & Kamarudin, 2016).
Regarding the diagnosis of Brucellosis in animals, it is based on the described symptommatology, corroborating with data related to the livestock - if new animals were introduced, their area of origin in relation to brucellosis cases. For confirmation, blood samples are collected that will undergo laboratory investigations by bacteriological, serological and allergic method (Zamri-Saad & Kamarudin, 2016).
Brucellosis is one of the diseases for which annual serological tests are required (Corbel, 1997):
- for all sheep and goats over 6 months old;
- all rams and rams for reproduction before the breeding season;
- all animals introduced into the farm;
- 5% of the flock of sheep and 5% of the flock of goats existing in non-professional holdings;
- all cattle over 24 months old;
- all breeding bulls and buffaloes.

The tests performed for brucellosis surveillance are the slow agglutination reaction, immunoenzymatic test - ELISA, polymerase chain reaction - PCR, fluorescent evaluation in polarized light - FPA, complement fixation reaction - CFT, the pink Bengal test - RBT, the ring test performed on the milk sample - MRT (Solera, 2000).
In humans, the diagnosis is based on symptoms corroborated with laboratory tests used to detect *Brucella* spp. bacteria, for a definite diagnosis, in blood or other biological samples such as bone marrow, urine, cerebrospinal fluid - CSF, synovial fluid etc. The most used serological tests are the agglutination reaction, the complement fixation reaction and immunoenzymatic. The first two types detect specific antibodies, to confirm the diagnosis it is necessary to demonstrate significantly increased titers, while the immunoenzymatic tests detect IgG and IgM antibodies, necessary to differentiate acute from chronic infection (Solera, 2000).
An epidemiological investigation is also carried out in which it is followed whether the patient had possible contact with infected animals or consumed products contaminated with *Brucella* bacteria (Solera, 2000; Radwan, 1993).
In patients suspected of brucellosis, the differential diagnosis is (Solera, 2000):
- flu;
- leptospirosis;
- malaria;
- meningitis;
- viral hepatitis;
- enteric fever;
- acute epididymitis;
- urinary tract infections.

In animals confirmed with brucellosis, the prognosis is unfavorable both for the infected animal and for the entire farm because they do not undergo treatment. Infected animals are slaughtered and their corpses are destroyed, and the rest of the animals are put under surveillance for 30 days. There is no treatment, the disease is kept under control by vaccination (Alavi & Alavi, 2013).
In patients confirmed with brucellosis, the treatment consists in the administration of antibiotics chosen according to the severity of the disease and the strain of brucellosis involved. The most common antibiotics are doxycycline, rifampicin, streptomycin, trimethoprim-sulfamethoxazole. In addition to these, medicines can be administered to combat fever and joint pain (Akova et al., 1993; Colmenero, 1989).
In brucellosis-free areas, one of the safest methods of preventing the spread of this pathogen is the close supervision of the areas of origin of animals newly introduced to the farm in relation to brucellosis cases. Ideally, they should come from brucellosis-free areas. Also, to control brucellosis, the following measures are required (who.int.2020; Smirnova et al., 2013):
- avoiding contact between sick or suspected disease animals and healthy ones;
-careful supervision of animal movement;
- medical supervision and isolation of animals at the manifestation of any symptom;
- arranging different spaces for pregnant animals;
- proper hygiene on the farm, especially after farrowing;
- the purchase of animals from disease-free areas;
- performing serological tests and quarantine for 60 days before introducing new animals into the herd;
- immunization of farm animals;
- the annual performance of mandatory analyses;
- the prohibition of the purchase of animals from areas with brucellosis;

- straw, garbage or any other objects with which animals infected with *Brucella* spp. came into contact are immediately destroyed, burned or buried after sprinkling with disinfectant;
- disinfection of paddocks, shelters, equipment before introducing other animals;
- means of transport, containers are cleaned and disinfected after transporting animals from an infected herd.

In humans, to control brucellosis, some important measures are necessary, such as (dambovita.dsvsa.ro; Alavi & Alavi, 2013):
- avoiding contact with infected animals or observing minimum protective measures such as wearing gloves, mask and protective equipment;
- rigorous personal hygiene measures such as washing hands and disinfecting surfaces that have come into contact with animals or products from infected animals;
- consumption of safe products, from healthy animals, properly pasteurized milk;
- vaccination against brucellosis of people who work with animals.

In 2021, 165 cases were confirmed in EU member countries, with a global rate of 0.04 per 100,000 inhabitants. Of these, France, Italy, Spain and Greece had the highest number of reported cases, 67% of all confirmations in EU member states. Luxembourg and Cyprus each reported one confirmed case, but the low population in these two countries resulted in relatively high notification rates. In Sweden, all confirmed cases have travel history to countries with brucellosis cases (Ecdc.europa.eu, 2021).

Also, in 2021, the lowest number of cases was reported in February, and the peak was reached in May-June. Of the total number of confirmations, 61% are for men and 39% for women (Ecdc.europa.eu, 2021).

In Romania, the last outbreak of brucellosis in animals was liquidated in 1959, since then it has remained silent regarding the infection with *Brucella* spp. (Ecdc.europa.eu, 2021).

Brucellosis is a disease with a great economic impact, because the losses it causes can be very high through abortions, morbidity after parturition, the reduction of productions, expenses related to the eradication of the disease, disinfection and surveillance (Akova et al, 1993).

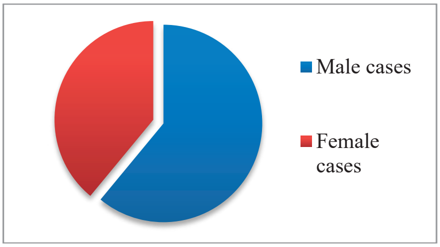

Figure 1. The graph of the percentage difference between the cases reported in men and women (Source: ecdc.europa.eu, 2021)

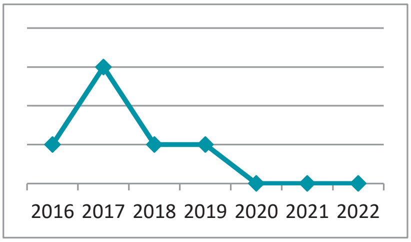

Figure.2. The evolution of human brucellosis cases in Romania (Source:eur-lex.europa.eu, 2016-2022)

Table 1. Number of confirmed brucellosis cases and notification rates per 100 000 population by country and year, EU/EEA, 2017-2021

Country	2017		2018		2019		2020		2021	
	Number	Rate	Number	Rate	Number	Rate	Number	Rate	Number	Rate
Austria	6	0.07	7	0.08	6	0.07	8	0.09	6	0.07
Belgium	8	0.07	9	0.08	3	0.03	4	0.03	7	0.06
Cyprus	0	0.00	0	0.00	0	0.00	0	0.00	1	0.11
Denmark	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Finland	1	0.02	0	0.00	0	0.00	0	0.00	0	0.00
France	21	0.03	0	0.00	34	0.05	19	0.03	21	0.03
Germany	41	0.05	37	0.04	36	0.04	19	0.02	13	0.02
Greece	94	0.87	97	0.90	65	0.61	30	0.28	24	0.22
Hungary	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
Italy	99	0.16	94	0.16	49	0.08	18	0.03	32	0.05
Luxembourg	0	0.00	0	0.00	0	0.00	0	0.00	1	0.16
Netherlands	2	0.01	5	0.03	7	0.04	2	0.01	2	0.01
Poland	2	0.01	0	0.00	2	0.01	0	0.00	1	0.00
Portugal	16	0.16	19	0.18	33	0.32	9	0.09	10	0.10
Romania	3	0.02	1	0.01	1	0.01	0	0.00	0	0.00
Spain	63	0.14	40	0.09	20	0.04	10	NR	25	NR

Source: Ecdc.europa.eu -Country reports; ND: no data reported; NR: no rate calculated

CONCLUSIONS

For free countries, the most important thing is to maintain freedom, especially through strict supervision of the import of animals and their own livestock.

It is one of the most important zoonoses and at the same time one of the most feared occupational diseases.

In animals, brucellosis colonizes the reproductive organs of host animals, causing abortions and sterility. They are shed in urine, milk, placental fluid and other animal fluids.

There is no treatment for brucellosis in animals. Vaccination of cattle, goats and sheep is recommended in enzootic areas with high prevalence rates. Serological or other testing and culling may also be effective in areas of low prevalence.

In cattle, it is caused by *Brucella abortus*, while in goats and sheep by *Brucella melitensis*, this being the main cause of brucellosis in humans.

For humans, the source of infection is represented by sick animals, products from infected animals or the infected environment.

Brucellosis is more common in men than in women, this fact also due to professional exposure to animals. In terms of age, brucellosis is more prevalent in the young population, with 60% of cases in people aged 13-40, 16% in those aged 40-60 and 2.5% in people 60 years and over.

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THE HISTORY AND THE MORPHO-PRODUCTIVE CHARACTERS OF THE BROWN CATTLE BREEDS

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Abstract

The theories underlying the studies on the origin of the cattle breeds place the Brown breed in the Bos taurus brachyceros type, based on the craniological characters identified in cattle from the mountain area. Today, at European level, a distinction is made between Braunvieh (original Braun-Schwyz) and Brown Suiss. The Braunvieh breed, a breed with milk-meat skills, was formed in the canton of Schwyz in Switzerland, based on successive crosses between local cattle and those brought by the Burgundians, Germans and Romans, later spreading to many countries in Europe. The Brown Suiss breed, specialized breed for milk, was formed in America, based on cattle imports from Switzerland (1869 - the first imports). While the Brauvieh breed achieves milk production of approximately 6,000 kg per lactation, with 4% fat and 3 .4% protein, the Brown Suiss breed, which has become a breeder for milk production, has achieved yields of over 10,000 kg of milk per lactation in the US. The proportion of beta casein type A2 (which gives better digestibility to milk) is much higher in the milk of these breeds compared to other breeds (over 80% in Brown Suiss compared to Holstein 62%).

Key words*: Brown cattle breeds, Braunvieh, origin, performance.*

INTRODUCTION

Looking to the tendency of worldwide development of population and them consumption needs, dairy industry is trying to manage this objective more and amore (Franzoi et al., 2020). In this dynamic atmosphere, the breeding and enhancement of cattle breeds become essential to meet demand and ensure sustainable and efficient production (Zhao et al., 2015). Moreover, in the quest to optimise yield and milk quality, numerous genetic improvement programs have been initiated globally (Mapel et al., 2022; Pacheco et al., 2023). These programs target the selection and improvement of morpho-productive traits of cattle breeds, including milk production, fat and protein content, disease resistance, and adaptability to various environments (Pacheco et al., 2023). Thus, genetic aspects of cow breeds could be considered, to identify diversity even within the same breed (Moscarelli et al., 2020). A representative breed which is present all over the world, and has an important place in cattle population, is figured by the Brown cattle breed (Unal et al., 2019). Being a popular breed for both industries, beef and dairy (Bozkurt & Dogan, 2019), the distribution of the Brown Swiss or Braunvieh is worldwide, starting with Europe, America, Canada, USA, even in New Zealand and South Africa (The cattle site). Moreover, this breed has istorical relevance, its origin is attributed to the Alpine region of Switzerland, where it was first raised over 4000 years ago, where researchers demonstrate that prehistorical bones of cattle found in a lake, are similar with Brown cattle from today (Del Bo et al., 2001; Moscarelli et al., 2020). Since then, the brown breed has spread worldwide. However, at the European level, there are three different groups of brown cattle population, starting wih original Braunvieh, which is the oldest one, the Braunvieh, and of course the Brown Swiss (Hagger, 2005; Moscarelli et al., 2020). Furthremore, the productive longevity of the udder in Brown swiss is superior and durable, even compared to Holstein Friza (Gibson et al., 2018). Researchers such as Moscarelli et al. (2020) has shown that several factors (geography, environment, natural selection etc.) and practices (artificial insemination) affect the

genetics characteristics of Brown cattle breeds population.
The Brown cattle breed, originating from Europe and developed in the Swiss Alps region, represents a globally significant breed with a corresponding genetic heritage, featuring remarkable characteristics in acclimatization. It demonstrates efficiency in various cold, rugged, arid, and dry environments, at high altitudes as well as in warm environments with poor pastures. Possessing mixed production traits, it has been improved over the past 150 years for milk production, ranging from 2,000 kg milk/lactation (Turkey) to 17,188 kg milk/365 days (record holder Agata cow, Germany) (Georgescu, 1998). This cattle breed has an average feed intake of 1.1 U.N./kg of milk, exhibiting exceptional quality (B-type K-casein). The Brown breed also maintains a significant position in meat production, with a carcass yield of over 50%, potentially reaching up to 59% with a daily average gain of over 850 grams/day, up to 1200 grams/day with a specific intake of 5.5-6.5 U.N./kg gain. It stands at a wither height of 132-147 cm, with calves weighing 35-40 kg at birth (Georgescu, 1998).
Therefore, the objective of the present study was to highlight aspects of the evolution, as well as the historical spread of Brown cattle breed, including the morpho-productive characters.

MATERIALS AND METHODS

For the accomplishment of this study, a systematic looking for other relevant scientific articles on the chosen subject was conducted in the considerable publishing platforms, such as PubMed, Google Scholar, Frontiers and MDPI databases. Relevant articles published in recent years, in the filed of brown cattle breeds were examined, and the references of included studies were also consulted to acquare additional bibliographic sources.

RESULTS AND DISCUSSIONS

American Brown Swiss

The American Brown originates from the Schwyz breed, it appeared in the USA in 1869 in Massachusetts, where 6 Browns and a bull from the canton of Schwyz (Switzerland) were brought. The Americans, as with the Friesian breed, focused their attention on milk production, making intensive improvements in this regard in the USA. meat production was not of interest to this breed as it was satisfied by local breeds bred for this production. As results, we can mention the year 1953 when a number of 400,000 heads were registered in the genealogical register, this doubling in 1978 reaching a number of 830,000 females under observation. US transformed the mixed breed he had imported into a breed specialized in milk production appreciated and sought after worldwide (BSA, 2024).
The Brown Swiss benefits from a robust constitution, fertility and precocity being at high levels, the temperament is lively, the character is gentle showing health and good adaptability to different climates. This breed stands out for its milk production achieving 6050 kg per lactation with 4.1%. As records we can remember at the age of 30 months a production of 7205 kg with a fat of 4.53%. The maximum production at that time was 15787 kg of milk with a fat content of 4.53% (Table 1), the first calving takes place at an age of less than 30 months, in the first lactation they produce somewhere at 75% of the maximum amount, we can also remember the uniformity of the milk. Consumption is 1 U.N./kg of milk with praiseworthy skills for mechanical milking. The Brown Cow can be found on the American continents, such as Mexico, Cuba, Panama, Costa Rica, Chile, Peru, Venezuela, Colombia, and Brazil (BSA, 2024).

Table 1. American Brown Swiss production for last 10 years (source: https://www.brownswissusa.com/)

Year	Milk quantity (kg)	Fat (%)	Fat (kg)	Protein (%)	Protein (kg)
2022	1175	4.09	480	3.34	392
2021	11575	4.04	468	3.33	385
2020	11444	4.03	461	3.32	380
2019	11811	4.02	455	3.34	377
2018	11391	4.07	463	3.33	379
2017	11430	4.05	462	3.32	379
2016	11441	4.03	461	3.31	378
2015	11231	4.02	451	3.32	373
2014	11114	4.09	454	3.34	370
2013	11143	4.03	449	3.31	368

Austrian Brown (Österreichisches Braunvieh)

These animals were bred and improved for the rugged areas of Austria. The Braunvieh shows great resistance of the legs, over 28% of the adult animals being grazed, mostly the youth reaching the alpine pastures. The Austrians believe that this breed cannot be beaten by any other in terms of its endurance in transhumance. Through its long productive life, it becomes a fairly economical breed. Regardless of the pressure placed on this animal, both in terms of production and the maintenance system (BSAA, 2024). The height at the withers is 132 cm, the average weight is 550 kg, the sexual dimorphism is quite pronounced, the bulls reaching a height at the withers of 138 cm with a weight of 850 kg. Milk production is around 4,000-5,000 kg. In Austria, for this breed, the percentage of foreign blood accepted was 12.5% until 2009, being a small population, the objective being to reach a percentage of foreign genes of 6.25% in 2014, all these in females. In Austria, we find a herd of 100,000 animals representing 6% of the total cattle herd in this country (BSA, 2024).

German Brown Swiss (Braunvieh)

The origin of the Brown breed dates back to the mountain areas of Switzerland, from where it was introduced to Germany in the 16th and 17th centuries. The breed adapted well to the mountain conditions of southern Germany and became popular for its hardiness, adaptability and milk production. In the 20th century, with changes in farming practices and the introduction of other specialized milk breeds, the Brown population in Germany declined.

The number in Germany is 350,230 animals of the Good breed, of which 163,726 are dairy cows, 146,965 are in milk production control (90%) (GGI-Spermex).

It is an animal with an enviable adaptability for areas lacking in the breeding of other breeds of cows, it is 142-155cm tall, weighs over 600 kg, reaches productions of 8,000-9,000 kg of milk with at least 7-8% fat and protein (GBSA, 2024).

The average milk production per lactation of the Brown breed in Germany is about 7,658 liters, the milk has a fat content of 4.25% and a protein content of 3.62% (GGI-Spermex).

The breed is valued for its milk quality, suitable for various traditional German dairy products, is used in small and family farms in mountain areas, for crossbreeding programs with other breeds to improve certain characteristics and last but not least as part of conservation initiatives native races and the countryside (GGI-Spermex).

Bruna de los Pirineos (Spanish Brown Swiss)

This breed results from the fusion and absorption of local Catalan breeds, the first Brown Swiss imports took place in 1880 without being recorded in any writing. In 1922 with the first registrations, the consolidation of the breed took place later until 1960. The Spaniards bet on meat production directing the breed in this direction. This breed is bred in an extensive system in its natural habitat, mainly in the Pyrenees area. It is appreciated for its average weight at birth (46.5 kg) which leads to ease of calving, the average daily gain is high, here we can also mention the increased fertility of these females. The product subjected to fattening can reach a weight of 540-550 kg at the age of 12-13 months, as reproduction, the mount is used, not using artificial insemination. The color is typical of Browns, with a height at the withers of 142 cm in males and 140 in females, males reaching a weight of 1,050 kg, females reaching a weight of 600 kg (BBLA, 2024).

Females reach sexual maturity at 24 months, males reach sexual maturity at 15 months, and they are used for reproduction on average until the age of 69 months. The first calving is recorded at 33.5 months, with an interval between calvings of 385 days, the number of calvings per year is 0.95. Females are exploited for an average of 9 years (BBLA, 2024).

Meat production, fattened calves have an average daily gain of 1300 gr, the average slaughter age is 12 months reaching a carcass weight of 270 kg with a yield of 61% (BBLA, 2024).

On 31.12.2022 in Spain we found a number of 3,682 females, in the same reference date we can remember a number of 3,708 calves. The number of purebred females is 687 heads, with only 646 registered in the breed book. In the mountainous area, this race has a responsibility of 75%. The total number of browns is 17,057 with 2,492 breeding males. The distribution area being Barcelona, Girona, Lleida (BBLA, 2024).

Table 2. Average national lactations/305 days in Spanish Brown Swiss (2020) (source:https://www.mapa.gob.es/es/)

Total	Nr. of lactations	Milk kg	Fat kg	Fat %	Protein kg	Protein %
First lactation	432	7351	301	3.88	274	3.60
Second and thirs lactation	938	7696	326	3.82	297	3.57
All animals	1370	7524	314	3.85	285	3.58

Brazilian Brown Breed (Raça Pardo Suíço)

The Brown breed race in Brazil it was first introduced at the beginning of the 20th century, appearing in 1905 (FAO, 2024). Although not as popular as other cattle breeds in Brazil, the Brown breed is valued for its adaptability to the tropical climate, milk production and carcass quality (https://www.fazentatamandua.com.br/)

The Brazilian Brown breed has an average milk yield per lactation of around 4,000-5,000 litres, with a fat content of 3.8-4.0% and a protein content of 3.2-3.4%. The breed is valued for the quality of its milk, which is ideal for the production of cheeses and other dairy products (Carneiro & Lush, 1954).

Turkish Brown Breed (Montofon)

It was first introduced at the end of the 19th century. The breed has gained popularity in regions of the country due to its adaptability to the local climate, milk production and carcass quality. The Turkish Brown breed has an average milk production per lactation of around 5,000-6,000 litres. milk has a fat content of 3.8-4.0% and a protein content of 3.2-3.4% (TBSA, 2024). The breed is valued for the quality of its milk, which is ideal for the production of cheeses and other dairy products. The meat obtained from cows of this breed is also of good quality. The Brown breed in Turkey is mainly used for milk production (TBSA, 2024).

The reproduction of this breed in Turkey, has an early sexual maturity, with an average age at first calving of 24-26 months, the average interval between calving is 12-14 months, the fertility of the breed is good, with a conception rate of 80-90%. The number of animals is about 50,000 cows in Turkey. The breed is concentrated in the mountainous and submontane areas of the west and north of the country (TBSA, 2024).

Rusian Brown Swiss (Shvitskaya)

The first series of Brune was imported to Russia from Switzerland in 1861, on a farm of the Moscow Agricultural Academy. This breed quickly became popular in the Moscow, Smolensk and Tula regions, as well as in other parts of Russia. Imports of genetic resources continued in the 1920s and 1930s, also from Switzerland, and between 1958 and 1972 there were imports from Austria, Switzerland, Hungary and the USA. Later, importation of semen and bulls from USA and Canada continued (BSRF, 2024).

The Russian Brown breed has an average milk production per lactation of around 5,000-6,000 litres. Milk has a fat content of 3.8-4.0% and a protein content of 3.2-3.4%. The Russian Brown breed has an early sexual maturity with an average age at first calving of 24-26 months, Average calving interval is 12-14 months, breed fertility is good with a conception rate of 80-90% (BSRF, 2024).

The Russian Brown breed is mainly used for milk production and is valued for its adaptability to various climates, being an important option for farmers in areas with extreme temperatures. The meat obtained from cows of this breed is also of good quality (BSRF, 2024).

According to the statistics of 2023, the total cow population in Russia was about 20.8 million, assuming that the brown Swiss breed represents 0.93% of the total, this would result in a number of about 205,920 head of brown cows (BSRF, 2024).

Table 3. Production and reproduction data at Russian Brown (2020)
(source: https://www.fao.org/dad-is/browse-by-country-and-species/en/)

Average milk production	6221 kg
Milk Fat	4.03%
Milk Proteine	3.34 %
Farms milk average	13,000-14,500 kg
Female weigh	570-600 kg
Male weigh	900-1000 kg

Italian Brown Swiss

The Italian Brown Swiss breed has a population of around 500,000 animals. More than 8,000 dedicated breeders participate in selection programs, raising an average of 21 animals per farm (14 cows). In Italy, it is used both for meat and milk production (ANARB, 2024). The mean national output of the cohort of 104,000 managed bovines amounted to 6,954 kg, exhibiting a consistent annual increment of 100 kg over a decade. This output is characterized by a protein content of 3.50% and a fat content of 3.96%. Notably, within this population, there exist 20 elite herds demonstrating superior performance, boasting an average yield of approximately 10,607 kg coupled with an average protein content of 3.69% (ANARB, 2024).

Romanian Brown Swiss (Maramures Brown Swiss)

It was formed in the north of the country, in the current territory of Maramureș County. the first browns were brought from Allgau Germany, around the towns of Sighet and Vișeu where cows from the Schwyz breed were brought, thus starting the absorption of local breeds. In the 1890s and 1910s cows were still imported from Austria and Germany, in 1904 imports were also made from Switzerland, with up to 500 Schwyz cows and heifers being brought annually (ANARZ, 2024). In the 1920s bulls were imported. In 1930, the first Schwyz cow farm was established in the commune of Beclean, Romania. Between the years 1948-1949, 119 breeding bulls and 700 pregnant heifers were imported, these imports being made by the former I.A.S. from Maramureș, part of the bulls being distributed in some counties in the Subcarpathian area of Muntenia and Moldova. Another variety (the Allgau variety from Bavaria) of the Schwyz breed was brought to the north of Muntenia between 1900 and 1910, from Germany (ANARZ, 2024). The Brown race began to expand in the rest of the country, in the southern area, in Argeș County, Vâlcea, Mehedinți, Prahova, Buzău, Gorj. During the expansion of the breeds, the degree of interbreeding was very different due to the local breeds that were in the respective areas, but also to the different climatic conditions, the various forms of forage, the maintenance conditions and last but not least the interest in breeding. At the end of 2000, the Brown breed was found in a percentage of 29.8% of the total bull herd in Romania, with an increase of 1-2% every 5 years. currently at a standard production of 5 097 kg, with frequent production limits between 3,000 and 6,000 kg/lactation (ANARZ, 2024).

Table 4. Average milk production in Romanian Brown Swiss (source: https://www.anarz.eu/)

Average milk production						
Farm number	Ended lactations	Milk kg	Fat		Protein	
			kg	%	kg	%
15237	12093	5453	214	3.92	182	3.35

These animals have a good feed utilization capacity, on average for one liter of milk a Brown breed cow needs 0.8 and 1.3 U.N., with a protein intake of 95,100 digestible crude protein, for each nutritional unit (ANARZ, 2024). Here we can also mention the meat production which can be neglected, the bulls can reach the age of 12 months at a weight of 360 kilograms, achieving an average gain of 990 g/day with a slaughter yield of 54-55% obtaining a prized meat (ANARZ, 2024).

Figure 1. Natural pasture of a Brown Swiss cattle from Romania (source: own source)

It lends itself to all exploitation systems, being well adapted to environmental conditions and resistant to diseases. In the case of the male youth, at calving it reaches 30-35 kg, and at 12 months it can exceed 320 kg with weight gains of 0.8-0.9 kg, in semi-intensive conditions, with a consumption of about 7 UNC/kg.

CONCLUSIONS

In conclusion, Brown swiss cattle breed is spred all over the world, adapting its morpho-productive characteristics depending on the country of origin. Brown swiss is one of the oldest breeds of cows, first registered in Switzerland, thus becoming their autochthonous breed of cows. This breed lends itself well to both meat and milk production, being a resistant breed.

By deeply understanding the morpho-productive traits and history of Brown swiss cattle breed, we can direct our efforts towards the sustainable improvement of the dairy industry and animal welfare.

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EVALUATION OF ALTERNATIVE PORK PRODUCTION SYSTEMS ON PASTURE FROM A MULTIPLE PERSPECTIVE: WELFARE, ECONOMY, AND ENVIRONMENTAL PROTECTION

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Abstract

Integrated professional swine farms produce enormous amounts of manure, wich has become a source of environmental pollution. Diversification of production systems, encouragement of alternative exploitation on free land, which contributes to changes in growth and exploitation, lower socio-economic and environmental impact, production with reduced consumption of resources without loss of nutrients and conservation of biodiversity through sustainable development of areas, can be solutions that can reduce pollution. The measures proposed for implementation, to reduce the environmental risk, refer to methods and techniques of statistical filtering and spatial grouping based on the density of swine, well-being, the level of accessibility of food and fodder resources, the level of accessibility of meadows for own pork production and the level of environmental pollution according to nitrogen (N) and phosphorus (P) excretion.

Key words*: environmental risk, exploitation system evaluation, pasture, swine.*

INTRODUCTION

The economic-social importance of raising pigs for meat production resides in the fact that the species, no matter the production system, is valuable from the perspective of obtaining, in conditions of economic efficiency: fresh meat and meat preparations - bacon, ham; fats (lard); edible organs (liver, heart); other raw materials for industry (skin, hair).

Pigs are raised in almost all areas of the Earth and almost everywhere nationally, but areas with intensive corn crops are the main areas for raising pigs. Most pig production in Romania has shifted from extensive systems with open pens in closed, mechanized facilities – industrial-type farms, where exploitation requires the provision of microclimate conditions, welfare and nutrition control, and the resulting manure is a problem for the quality of the building environment if not well managed. The efficiency of production, the increasing need for pork offal means that, because of environmental problems, the classical exploitation systems have been diversified using new alternative exploitation systems in the open air and on pasture (Delsart et al., 2020), with the control of environmental factors and of the degree of supportability of the exploitation area (Giraldi-Díaz et al., 2021; Abrantes Pinto de Brito et al., 2022).

MATERIALS AND METHODS

The evaluation of systems for the exploitation of pigs on pasture for meat production involves the use of methods and techniques of statistical filtering and grouping of the density of pigs to ensure animal welfare and reduce the degradation of meadows according to the degree of supportability of the pasture, by controlling the level of pollution for maintaining biodiversity (Ruckli et al., 2021). The alternative system in open air in group pens with density control are:

• S75H/5B_15H - 75 heads kept in 5 boxes of 15 heads with a useful area of 0.80 m^2;

• S75H/5B_16H - 75 heads kept in 5 common boxes of 16 heads with access to the paddock with a useful surface of 0.70 m^2 per pig.

The statistical parameters evaluated are:

• AWupon entrance - average weight upon entrance in the farm;

• TWupon entrance - a total weight upon entrance in the farm;
• TWupon delivery - a total weight upon delivery to the slaughterhouse;
• AWdaily gain - an average daily gain over 95 days of fattening. The evaluation of the implemented alternative production system (Delsart et al., 2020) was done according to the Krieter method (2002) from a multiple perspective - welfare (environmental conditions), economy, and environment by measuring nitrogen and phosphorus excreta on the pasture (Chen et al., 2020). Monitoring of nitrogen and phosphorus excretion was determined using a nitrogen and phosphorus mass balance based on feed ration, crude protein content of the diet, total phosphorus and animal performance - reduction techniques by feeding in two meals with the provision of a diet food adapted to the requirements for fattening ages (PB = 14% up to 70 kg; PB = 12% until slaughter). Managerial measures were proposed to contribute to the reduction of losses of nutritional elements, by controlling risk factors and implementing the most effective alternative systems for obtaining meat by implementing policies to stop the development of farms and increase individual productions, ensuring welfare conditions for fattening pigs, economic efficiency of exploitation and protection of environmental factors, through the management system of alternative meat production in an integrated system, environmental and total quality management being important parts of technological management (Radhakrishnan et al., 2018; Andretta et al., 2021; Abrantes Pinto de Brito et al., 2022).

RESULTS AND DISCUSSIONS

Although pigs, depending on the production system and on its efficiency, can be fed any type of feed, in intensive and super-intensive farming, maintenance conditions, microclimate, and nutritionally balanced feeding must be ensured to produce fast and healthy growth, with a reduced amount of manure, which is the biggest problem of large holdings (Giraldi-Díaz et al., 2021). Used as granules, flours, dry or wet feed based on mixtures of corn and soybean meal supplemented with antibiotics, minerals, and vitamins (Sampath et al., 2023), feed can contribute to shortening the fattening period and increasing individual productions.
Ensuring welfare conditions in any production system contributes to obtaining high-performance productions with high costs without being able to fully solve the problems related to natural environmental factors (Figure 1).

Figure 1. New directions and changes in pig farming and sustainable development

For these reasons, we propose new solutions for the diversification of the meat production systems, for production efficiency and reduction of the degree of pollution by switching as much as possible from classic energy-consuming systems, where there are local resources to exploit on pastures and in the open air through:
- implementation of spatial planning of pig production in areas with:
• high availability of local fodder resources;
• availability of resources from pastures and arable land;
- implementation of community policies to reduce environmental risk through:
• the integration of environmental management into the integrated management of meat production (Radhakrishnan et al., 2018; Andretta et al., 2021; Abrantes Pinto de Brito et al., 2022);
• the development of the best practices that contribute to the preservation of biodiversity around pig farms (Ruckli et al., 2021; Teodorescu et al., 2023);
- implementation of new managerial measures as principles of action adopted in farms or proposed by product organizations or farmers in three periods of development of classical and alternative production systems (Delsart et al., 2020):

• unmanaged development for small professional meat farms with regulations regarding the welfare and environmental protection of their area; environmental regulations (1961-1978);
• rapid development of medium-sized professional farms that have implemented alternative outdoor and pasture production systems with:
• welfare measures;
• regulation of livestock depending on the creditworthiness of the meadow and on the degree of affordability;
• the possibility of securing resources in crisis conditions;
• the effects on the vegetative carpet, soil (Loss et al., 2019; Yost et al., 2022), and water;
• the possibilities of monitoring environmental factors (Andretta et al., 2021; Abrantes Pinto de Brito et al., 2022);
• stagnation of the development of large farms and the diversification of production systems, through:
• increasing individual productions, using commercial breeds and hybrids with high biological value;
• ensuring microclimate conditions and maintenance;
• implementing the best management of nutrition by increasing the efficiency of feed use and by producing reduced amounts of manure;
• implementing environmental risk management regulations (Radhakrishnan et al., 2018; Machete & Chabo, 2020);
• monitoring livestock production and controlling the degree of pollution in the farm area;
• integrating production, processing and capitalization by perfecting marketing management and production according to the trend of the pork market;
• transition to a more efficient alternative production with the consumption of local resources and environmentally-friendly by implementing the following measures applicable to any farm that adopted the alternative production system on pasture or in the open air by (Delsart et al., 2020; Andretta et al., 2021; Izmaylov et al., 2022):
• implementation of statistical filtering techniques of the pork demand from the potential market;
• density-based spatial clustering of noisy applications (Liu et al., 2023);
• accessibility to fodder and arable land for own production (Govoni et al., 2022);
• population migration (Bai et al., 2019) and consumption trends towards other types of meat and meat preparations;
- food accessibility level:
• traditions regarding the consumption and method of preparing meat;
• distribution and capitalization possibilities;
• the price of meat obtained in alternative systems;
• average level of income;
- pollution level and the negative impact on the environment in the farm area (Andretta et al., 2021; Abrantes Pinto de Brito et al. 2022).

If modern pig farms produce large amounts of manure, which has become increasingly worrisome as a potential source of water, soil (Loss et al., 2019; Yost et al., 2022), and air pollution, for sustainable development (Deviney et al., 2021) we propose other measures to diversify production systems based on the importance of socio-economic aspects of raising and exploiting pigs for meat production, emphasizing that it is a much more complex relationship regarding welfare, efficiency of exploitation, and the degree of pollution of environmental factors (Andretta et al., 2021); therefore, it is necessary to control the following influencing factors of production efficiency in classical systems and alternative systems for the new orientations in obtaining pork, preserving biodiversity, and sustainable development of the areas in the vicinity of the farms (Delsart et al., 2020; Deviney et al., 2021; Giraldi-Díaz et al., 2021; Ruckli et al., 2021):
- absence of available resources on pasture or agricultural land;
- presence of farms with plant and animal production;
- location of pig production as close as possible to the pork markets;
- ensuring the necessary quantities of food;
- ensuring food security and safety;
- demand, income level, and economic and energy crises;
- social economy and epidemics of infectious diseases that affect people (COVID-19) (Teodorescu et al., 2023);
- massive epidemics of infectious diseases affecting pigs (influenza, rubella, classical and

African swine fever) (Jarynowski et al., 2019; Berends et al., 2021);
- manure recycling facilities (optimized treatment, transport, and application);
- implementation of technological innovations (genetically modified animals, animal cloning, gene/genome editing, genetic engineering) along the entire pork production chain (Wu & Bazer, 2019);
- level of cereal imports (basic fodder in the pigs' diet);
- average income (which determines the increase in the demand for animal products);
- culinary habits, tradition, and dietary preferences (which depend on the level of education of the population, the average income, and urbanization rate);
- urbanization rate (city dwellers consume more pork than villagers).

Pigs operated in an alternative system in open air in group pens with density control achieved the following performances in common boxes with access to the paddock:

- 75 heads kept in 5 boxes of 15 heads with a useful area of 0.80 m^2 per head reached:
    - an average weight upon entrance in the farm of 24.60 ± 1.35 kg (Table 1);
    - a total weight upon entrance in the farm of 1,845.00 kg (Table 1);
    - a total weight upon delivery to the slaughterhouse of 7,970.50 kg (Table 1);
    - an average daily gain over 95 days of fattening of 859.72 g (Table 1);

Table 1. Performances obtained by pigs operated in alternative open-air system in group pens with density control in common stalls with paddock access

Evaluated parameters	Alternative system	
	$S_{75H/5B\_15H}$ (0.8 m^2/head)	$S_{75H/5B\_16H}$ (0.7 m^2/head)
$AW_{upon\ entrance}$	24.60±1.35 kg	24.51±1.44 kg
$TW_{upon\ entrance}$	1,845.00 kg	1,835.25 kg
$TW_{upon\ delivery}$	7,970.50 kg	7,901.20 kg
$AW_{daily\ gain}$	859.72 g	851.35 g

- 75 heads kept in 5 common boxes of 16 heads with access to the paddock with a useful surface of 0.70 m^2 per pig reached the following production parameters:
    - an average weight upon entrance in the farm of 24.51 ± 1.44 kg (Table 1);
    - a total weight upon entrance in the farm of 1,835.25 kg (Table 1);
    - a total weight upon delivery to the slaughterhouse of 7,901.20 kg (Table 1);
    - an average daily gain over 95 days of fattening of 851.35 g (Table 1).

To note that ensuring a density of 0.70-0.80 m^2 per head of animal during the fattening period ensures, through balanced nutrition, similar production performances but lower at higher densities. We conclude that, for alternative outdoor production systems, to ensure animal welfare we need:
- a minimum of 0.80-0.85 m^2 per head of animal because production performance is lower in smaller areas;
- access to the paddock and optimal conditions in the stalls, possibilities to adjust the environmental factors (Andretta et al., 2021) and to improve the efficiency of fattening by supplementing the rations with green fodder that is cheaper than the concentrated ones;
- noise reduction from feed conveyors and huma sources, and lower densities based on sound biological and economic research can help reduce feed consumption, as dietary vitamin supplementation reduces stress and improves welfare in fattening pigs;
- fitting the temperature during the fattening period because of its effects on animal welfare and on fattening through:
    - discomfort: disorganization of the stalls, dirtiness, crowding, lack of rest, altercations and exits from the herd through accidents and death;
    - low economic yields because of the spread of pathogens, appearance of technopathies, depreciation of carcasses and meat;
    - reduced yields at slaughter and unsatisfactory financial results;
    - impact on markets through reduced supply of meat obtained in an alternative production system;
    - failure to meet consumption needs on the pork market.

To maintain animal welfare and obtain economic productions that meet the needs of pork consumers on the market, we recommend the development of alternative production systems on pasture and in the open air and the monitoring and control of:
- the operating environment;
- optimal densities;

- the nutritional quality of fodder resources;
- modelling of resting, feeding, and watering places;
- harmonization of production parameters control systems;
- monitoring of environmental factors to preserve the biodiversity of pastoral ecosystems (Andretta et al., 2021; Ruckli et al., 2021).

Regarding meat production in open-air alternative system, environmental issues were monitored by controlling nitrogen (N) and phosphorus (P) excretion (Chen et al., 2020). Research results showed the following (Figure 1):

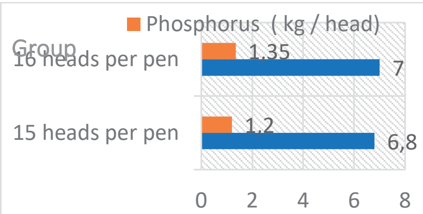

Figure 1. Results obtained by controlling the excretion of nitrogen and phosphorus, with the aim of monitoring environmental problems, in relation to meat production in the alternative system in the open air

- for the group of 15 heads per pen, nitrogen and phosphorus excretion was 6.8 and 1.2 kg, respectively, per pig head;
- for the group of 16 heads per pen, nitrogen and phosphorus excretion was 7.0 and 1.35 kg, respectively, per pig head;
- farming on pasture with the optimization of fattening pig herds and the increase of herds to the degree of supportability increases the welfare score by up to 25% and the cost, the excretion cost, and the excretion of N and P were reduced by 3.50-5.20;
- by monitoring environmental factors, optimizing herds fattening on pasture and using good practices, the risks of managing the natural environment can be reduced (Andretta et al., 2021). For the alternative systems of obtaining meat from the traditional Romanian pig breeds Negru de Strei, Mangalița, and Bazna, in areas with tradition, we propose for implementation a method of assessing the sustainability of pork production systems and practices considering 6 elements (Table 2):
- animal welfare;
- economy of resources, including the production cost;
- quality of the environment, perception, and human culture;
- solving the meat deficit;
- animal health and worker safety;
- food safety.

Table 2. Comparisons regarding the sustainability of classical and alternative production systems

Item	Pig production system	
	inside	outside
Zoonoses	controllable	predominant
Pork quality	good	very good
Environmental impact	0	0
Community interface	0	0
Perception of animal welfare	very good	very good
Productivity	100	20
Security of human resource	very good	good
Food safety	very good	poor
Welfare	very good	good
Climate variability	controllable	uncontrollable

The comparison between the classical and alternative systems shows that food safety concerns make the pig production system in alternative outdoor and pasture systems unable to cover the entire meat requirement but a niche of 10-12% making it unsustainable at the national level, though an important source of meat and meat products for isolated areas, where there is a tradition of consuming dry raw products with a long shelf life.

CONCLUSIONS

A few conclusions can be drawn from the presentation above:

✓ alternative farming systems are above classical farming systems;

✓ alternative farming systems are diversified (farming on straw to silvo-pastoral farming, free-range farming, organic farming);

✓ alternative farming systems differ to confined, conventional, slatted farming; alternative farming systems enjoy a very positive societal image;
✓ alternative farming systems have real strengths;
✓ alternative farming systems have weaknesses (animal welfare, economic profitability, farmer welfare, controlling biosecurity, sustainability).

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IN VITRO SCREENING OF LACTIC ACID BACTERIA AS BIOCONTROL AGENTS FOR BIOPRESERVATION OF PERISHABLE AGRO-FOOD PRODUCTS

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Abstract

The aim of this study was to evaluate in vitro the potential of different strains of lactic acid bacteria (LAB), belonging to Enterococcus faecium, Lactobacillus brevis, Lactobacillus farciminis, Lactobacillus fermentum, Lactobacillus plantarum, Pediococcus acidilactici, Pediococcus pentosaceus and Weissella cibaria, for their potential use for increasing the shelf life of different perishable food products. Several screening tests were taken into account, mainly related to their probiotic potential, but also the potential use as biocontrol agents during storage. The strains of P. pentosaceus, L. fermentum, and L. plantarum had the greatest biopreservation potential, with a spectrum of antimicrobial activity against a wide range of pathogenic bacteria and food spoilage fungi. They are capable of producing bacteriocins, exopolysaccharides, organic acids (lactic and acetic acids), enzymes (protease and phytase). In addition, freeze-drying with glucose as a cryoprotective agent resulted in a high survival rate of LAB strains, with a survival rate exceeding 50%. The aforementioned findings suggest that out of the LAB strains tested, L. plantarum MI207 represents a viable option for extending the shelf life of fresh, minimally processed food products in a sustainable manner.

Key words: *lactic acid bacteria, preservation, perishable food, probiotic.*

INTRODUCTION

In view of the demands of modern living and the resulting pressure on one's time, numerous studies have demonstrated that a daily diet rich processed and ultra-processed foods ready-to-eat, characterised by a high calories content and a low nutrients level, can lead to overeating and weight gain (Valicente et al., 2023; Barbaresko et al., 2024; Dicken et al., 2024). Over time, this may give rise to a range of health issues, such as obesity, type 2 diabetes, high blood pressure, heart disease, certain types of cancer, as well as a negative impact on mental health (Juul et al., 2022; Mazloomi et al., 2023; Valicente et al., 2023; Dicken et al., 2024). It is therefore strongly recommended that a diet comprising fresh and minimally processed foods, including vegetables, fruit, and whole grains, be adopted. Such foods provide essential nutrients, including vitamins, minerals, and dietary fibre, which are necessary for a healthy lifestyle. The question thus arises as to how these fresh or minimally processed foods can be kept safe for a longer period without the use of chemical preservatives?

The risk of pathogens' contamination can be reduced when fresh vegetables are processed using appropriate sanitation techniques. To remove soil, grime, debris, and potentially microorganisms from the surface of fresh products, decontamination techniques often involve washing the product with water or a solution of water and disinfectants (Machado et al., 2017; Huang et al., 2018; Yoon et al., 2018). However, these methods may not be sufficient to completely remove biofilm from fruit and vegetable surfaces.

The term "bioprotectant agents" is used in the context of food biocontrol and refers to live microorganisms and/or their metabolites that have been intentionally incorporated into food to inhibit the growth of undesirable microorganisms while preserving the sensory properties of the food. Lactic acid bacteria (LAB) are a group of non-pathogenic microorganisms (generally recognized as safe, or GRAS) (Axelsson, 2004; Shi et al., 2022). They have a long history of use as starter cultures and probiotics (Patarata et al., 2024). Their multifunctional properties have been exploited to develop a range of functional foods with high-value nutraceuticals and to promote health. LAB can convert carbohydrates into organic acids (mainly lactic acid) and produce a range of metabolites (e.g exoplisaccharides) that enhance the sensory and nutritional qualities of food, as well as antimicrobial components (i.e. bacteriocins) that prolong the shelf life of food products (Varsha et al., 2016; Agriopoulou et al., 2020; Peng et al., 2020; Bhattacharya et al., 2022; Zapaśnik et al., 2022).

While there has been a notable increase in the number of scientific papers published on the subject of utilising LAB species or their metabolites as biocontrol agents in fruit and vegetable preservation, there has not yet emerged a standardised methodology for their implementation (Corbo et al., 2015; Linares-Morales et al., 2018; Agriopoulou et al., 2020; Badea et al., 2022; Sri et al., 2023; Ramos et al., 2024).

The efficacy of LAB as biocontrol agents is affected by a multitude of factors intrinsic to the LAB agents themselves (e.g., specie/s and strain/s) and extrinsic factors (e.g., percent of inoculation (CFU/ml or g), the presence of associated pathogenic bacteria in microbial consortia, the presence of microbial biofilm, physical and chemical properties of the products; maintaining LAB viability during storage conditions, etc.).

Investigation of LAB isolated from indigenous foods and beverages and more could lead to the identification of previously unknown species or strains that may exhibit properties of biotechnological interest (Diguță et al., 2020; Daba et al., 2021, 2022; Pristavu et al., 2022; Ouili et al., 2023; Kouadio et al., 2024).

The genera *Bifidobacterium, Lactococcus, Lactobacillus, Leuconostoc, Pediococcus, Streptococcus,* and *Weisella* are well-known and extensively studied (Daba & Elkhateeb, 2020). LABs, either through the inoculation of viable cells and/or cell-free supernatants or through the incorporation of edible coatings, have been exploited to improve the shelf-life of fresh fruit and vegetables during storage while maintaining sensory properties (Iseppi et al., 2022).

This study targeted the *in vitro* evaluation of several LAB strains for their potential use to increase the shelf-life of different perishable food products and further development of edible coatings for different fresh fruits.

MATHERIALS AND METHODS

1. Lactic acid bacteria strains

A total of 15 LAB strains were used in this study (Table 1). All LAB strains were maintained at -20°C in MRS broth (Man, Rogosa and Sharpe, Oxoid, Limited, Hampshire, United Kingdom) containing 20% glycerol. For the pre-inoculum LABs were cultivated in MRS broth at 37°C for 24 hours.

Table 1. LAB strains used in this study

Species name	Code	Microbial collection
Enterococcus faecium	MI201	UASVM Bucharest
Lactobacillus brevis	MI202	UASVM Bucharest
Lactobacillus farciminis	MI203	UASVM Bucharest
Lactobacillus fermentum	MI204	UASVM Bucharest
Pediococcus pentosaceus	MI205	UASVM Bucharest
Weissella cibaria	MI206	UASVM Bucharest
Lactobacillus plantarum	MI207	UASVM Bucharest
Lactobacillus plantarum	MI208	UASVM Bucharest
Pediococcus acidilactici	MI209	UASVM Bucharest
Pediococcus pentosaceus	MI210	UASVM Bucharest
Pediococcus pentosaceus	MI211	UASVM Bucharest
Lactobacillus brevis	MI212	UASVM Bucharest
Pediococcus pentosaceus	MI213	UASVM Bucharest
Pediococcus pentosaceus	MI214	UASVM Bucharest
Pediococcus acidilactici	MI215	UASVM Bucharest

2. Antimicrobial activity

2.1. Antibacterial activity

Antibacterial activity was assessed against different representative pathogenic bacteria (*Escherichia coli* ATCC 25922, *Listeria ivanovii* ATCC 1911, *Listeria monocytogenes* ATCC 7644, and *Salmonella enterica* serovar Typhimurium ATCC 14028) using two distinct tests such as the dual culture plate assay of the LAB cells and the agar well diffusion assay of the CFSs (cell-free supernatants), as previously described by Balouiri et al. (2016) and Diguță et al. (2020) with minor modifications. Briefly, the reference bacteria were cultivated in trypticase soya broth (TSB, Alliance Bio Expertise, France) at 30°C, for 24 h. An aliquot (10 µL) of an overnight LAB culture was inoculated in a spot on MRS agar distributed on a Petri dish. The reference pathogenic strain was inoculated in Tryptic Soy Agar (TSA, Alliance Bio Expertise, France) maintained at 45°C, and spread over the initially inoculated MRS agar medium. The free-cell supernatant (CFS) was obtained from an overnight LAB culture via centrifugation (at 10,000 x g for 5 min, at 4°C) and filtration through sterile Millipore 0.22 µm filters (Sartorius, Goettingen, Germany). Wells with a diameter of 6 mm were punched aseptically with a sterile tip and filled with 100 µl of the tested CFS. Then, agar plates were incubated at 37°C for 24 hours. The inhibition zone, defined as the area surrounding each well and exhibiting an inhibition size of at least 1 mm, was indicative of the antibacterial efficacy of the LAB cells and the CFS.

2.2. Antifungal activity

The ability of LAB strains to inhibit fungal growth was evaluated against 10 pathogenic fungi, respectively *Aspergillus brasiliensis* ATCC 16404, *A. carbonarius* MI 15, *A. flavus* MI 24, *A. niger* MI 5, *A. ochraceus* MI 2, *Botrytis cinerea* MI Aligote Huși, *Fusarium oxysporum* MI 3, *F. proliferatum* MI 4, *Penicillium digitatum* MI 22, and *P. expansum* MI BB Husi. The antifungal activity was evaluated using a dual culture assay, as previously described by Diguță et al. (2020). An aliquot (10 µL) of an overnight LAB culture was inoculated in a spot on MRS agar distributed on a Petri dish and incubated for 24 h at 30°C. A fresh layer of potato dextrose agar (PDA, Alliance Bio Expertise, France), including a fresh fungal spore suspension (10^6 spores/ml), was then placed over the initially inoculated MRS agar medium. After another 48 h of incubation at 30°C, the size of the zone of inhibition that develops in the immediate proximity of the LAB strains was measured with a ruler.

3. On-plate testing of bacteriocin producing

The bacteriocin production of the LAB strains was determined using the method proposed by Matei et al. (2018). The LAB strains were grown in MRS broth for 24 hours at 37°C and then centrifuged at 5000 rpm for 10 minutes. The supernatant was subsequently collected and neutralised with a 40% sodium hydroxide solution. A volume of 3 µL of the supernatant was added to the MRS agar plate in a spot.

The bacteriocin-sensitive *Streptococcus thermophilus* ATCC 19258 strain was inoculated onto 20 mL of MRS agar maintained at 45°C and then poured into an initially prepared Petri dish. After 24 hours of incubation at 37°C, the appearance of an inhibition zone surrounding the spot indicated a positive result.

4. Exopolysaccharides production assay

LAB strains were inoculated onto MRS broth (containing 5% glucose) and then incubated at 30°C for 48 hours. The methodology used was described by Paulo et al. (2012), with some modifications. To highlight the production of the exopolysaccharides (EPS), the overnight LAB cultures were inoculated on MRS agar supplemented with 30% sucrose, as a carbon source.

After incubation at 30°C for 72 hours, the production of EPS was assessed visually by the formation of mucoid and translucent colonies on the agar plates. The slimy aspect was determined by gently touching them with a sterile inoculation loop.

5. On-plate screening of organic acids production

The production of lactic acid was tested following the methodology described by Kumar et al. (2020). The medium consisted of molasses (150 g/l), malt extract (2.5 g/l), yeast extract (2.5 g/l), peptone (2.5 g/l), and agar (15 g/l), which was distributed in Petri dishes. To enhance lactic acid production, calcium carbonate was incorporated as a neutralizing agent. LAB strains were inoculated in spots (10 µL) and then incubated at 30°C for 2-3 days. Lactic acid production was indicated by the formation of a clear inhibition zone surrounding the LAB colonies.

For the acetic acid, a volume of 10 µL of an active LAB culture was spotted on Chalk agar plates (comprising glucose (10 g/L), yeast extract (3 g/L), calcium carbonate (3 g/L), with agar (20 g/L) as the substrate), according to the methodology described by Sidari et al. (2021), with a few modifications. These were subsequently incubated at 30°C for five days. The presence and degree of a transparent halo surrounding the LAB strains provided evidence of the rate at which acetic acid was produced.

6. Detection and quantification of organic acids content by HPLC

The quantification of organic acids (lactic and acetic acids) was conducted via high-performance liquid chromatography (HPLC), with samples prepared by centrifugation and stored at -18°C. For subsequent analysis, samples were diluted with ultrapure water and then filtered through a 0.20 µM Millex PTFE membrane.

The chromatographic analysis was conducted using a Waters Alliance system, which includes a separation module and a UV detector (both manufactured by Waters; Millipore, Milford, MA, USA). The separation was conducted using two SUPELCOGEL H Guard Columns (250 mm x 4.6 mm and 50 mm x 4.6 mm) with 0.1% H_3PO_4 as the mobile phase. UV detection was performed at 210 nm. All data were analysed using the Empower 2.3 system (Waters Corporation, Milford, MA, USA). The quantification process was based on the peak area, with a calibration curve obtained by injecting different volumes of organic acid standard solutions.

7. On-plate screening of hydrolytic enzymes production

LAB isolates were inoculated in spots on the surface of culture media that had been supplemented with casein (for protease) and sodium phytate (0.2% w/v) (for phytase). The proteolytic activity was determined using culture media containing skim milk (PanReac Applichem, Darmstadt, Germany) in a 1:2 ratio with water (v/v) and 2% agar, as previously described by Sidari et al. (2021). Phytase activity was measured using a methodology previously described by Bhagat et al. (2020). The medium is composed of glucose (0.5%), peptone (1%), yeast extract (0.5%), magnesium sulfate (0.1%), calcium chloride (0.1%), and sodium phytate (0.2%) (Sigma-Aldrich, Missouri, USA). The plates thus prepared were incubated at 30°C for 96 hours. The presence of a translucent halo surrounding LAB strains that exhibited protease and phytase production was considered inductive of the desired outcome.

8. Antioxidant activity

The 1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity of the tested LAB strains was evaluated using the method previously described by Brand-Williams et al. (1995), with some modifications. Briefly, overnight LAB cultures were centrifuged at 10,000 x g for 5 minutes. 1 ml of a free-cell supernatant was vigorously mixed with 2 ml of 100 µM DPPH solution (dissolved in pure ethanol) (Aldrich, Merck KGaA, Darmstadt, Germany), and then incubated in a dark environment for 30 minutes at room temperature. After this incubation period, the mixture was centrifuged at 10,000 × g for 5 minutes. The absorbance of the supernatant at 517 nm (OD517) was measured utilizing a UV-1800 spectrophotometer (ChromTech, Minneapolis, USA). To ensure the accuracy and reliability of the results, the control samples were substituted with an equivalent volume of distilled water. In the blank sample, DPPH was substituted with an identical volume of absolute ethanol. An equal volume of distilled water and absolute ethanol was employed as a calibration standard, thus enabling the assessment of the accuracy and precision of the results. The percent of radical scavenging activity was estimated using the following equation:

$$\mathrm{AA}\ \% = \frac{\mathrm{ABSDPPH} - \mathrm{ABSsample}}{\mathrm{ABSDPPH}} \times 100.$$

where: AA(%) = antioxidant activity (%); ABS_{DPPH} = the absorbance of DPPH solution without any sample; ABS_{sample} = the absorbance of the mixture of 2 mL DPPH and 1 mL sample.

9. Preservation of LAB by freeze-drying procedure

The overnight LAB cells cultivated in MRS broth were harvested by centrifugation at 4000 x g for 10 minutes at 4°C. The cells were washed twice using PBS buffer (VWR Chemicals, Ohio, USA) and suspended in 2 ml of D-glucose (Carl Roth GmbH, Karlsruhe, Germany), which was used as a cryoprotectant agent.

Following freezing overnight at a temperature of -20 °C, the cell suspensions were freeze-dried in a chamber-type freeze-dryer (FreeZone6, LABCONCO, 6 L Benchtop Freeze Dry System, Kansas, MO, USA) at a temperature of -55 °C and an absolute pressure of 0.3 millibars for 4 hours.

Before and following the freeze-drying procedure, the plate count method was employed to assess cell viability. The survival rate of LAB strains was calculated according to the following formula:

$$\text{Viability } \% = \frac{\text{log CFU/ml after lyophilization}}{\text{log CFU/ml before lyophilization}} \times 100.$$

10. Statistical analysis

The obtained results are the average of three independent trials conducted under identical conditions. All data are presented as the mean ± standard deviation. The calculations, figures, and boxplots were performed using Excel 2019.

RESULTS AND DISCUSSIONS

1. Antimicrobial activity of LAB strains

All isolates exhibited moderate to high inhibitory activity against *S. enterica* Typhimurium, *L. monocytogenes,* and *L. ivanovii,* as detailed in Table 2. The majority of LAB cells displayed a comparable degree of inhibitory activity against *E. coli,* although the inhibitory effect appeared to be less pronounced in *L. brevis* MI202 and *P. acidilactici* MI215.

Overall, the antibacterial activity of CFSs was significantly lower than that of LAB cells (Table 2). However, the CFSs derived from *Ent. faecium* MI201, *L. brevis* MI202, and *W. cibaria* MI206 strains demonstrated moderate inhibitory effects against both *L. ivanovii* and *L. monocytogenes*. Moreover, the CFSs derived from the *P. pentosaceus* MI205 strain exhibited a strong inhibitory effect on *L. ivanovii* and *L. monocytogenes*.

In a study conducted by Trias et al. (2008), five LAB strains were identified as capable of inhibiting the proliferation of *L. monocytogenes* and *S.* Typhimurium in cut iceberg lettuce leaf. However, they did not demonstrate the same inhibitory effect against *E. coli*. Siroli et al. (2014) demonstrated that a nisin-producing strain of *Leuconostoc lactis* could inhibit the total mesophilic species, *E. coli*, and *L. monocytogenes,* when added at a level of 7 log CFU/ml in the washing solution of minimally-processed lamb's lettuce. *Pediococcus* strains isolated from the Komubucha consortium exhibited high antibacterial activity against *Bacillus cereus, L. ivanovii, L. monocytogenes, Proteus hauseri, S. enterica* Typhimurium, and methicillin-resistant *Staphylococcus aureus*, while exhibited low activity against *E. coli* (Diguță et al., 2020). A further study demonstrated the pronounced inhibitory effects of *Lactiplantibacillus plantarum* strains against *L. monocytogenes, S. enterica* serovar Typhimurium, and *S. aureus*, while their impact on *E. coli* was observed to be moderate (Kouadio et al., 2024). A cocktail of *L. plantarum* and *P. pentosaceus* significantly reduced *L. monocytogenes* and *Salmonella enterica* populations on artificially contaminated fresh strawberries for 7 days (Yin et al., 2022).

Filamentous fungi, particularly species from the genera *Aspergillus, Botrytis, Fusarium,* and *Penicillium*, are responsible for the contamination of fresh fruits and vegetables, resulting in significant economic losses.

Extensive research has demonstrated the inhibitory effect of LAB on the development of the filamentous fungi, due to the effect of their biosynthesized metabolites which disrupt the integrity of the fungal cell membrane and inhibit the uptake of amino acids by the fungus (Marie et al., 2018; Sadiq et al., 2019; Nasrollahzadeh et al., 20221; Shi et al., 2022).

In our study the lactic acid strains showed low antifungal activity against *Aspergilli* group. Only strains *L. brevis* MI212, *P. pentosaceus* MI211, *Ent. faecium* MI201, *L. farciminis* MI203 and *L. fermentum* MI204 showed inhibitory activity against *A carbonarius*; *L. brevis* MI202 and *L. fermentum* MI204 against *A. brasiliensis*, respectively. The highest inhibitory activity was shown by *L. brevis* MI212 against *A. carbonarius*. No other LAB strain demonstrated antifungal activity against *A. flavus, A. niger,* and *A. ochraceus*.

Table 2. Antibacterial activity of the LAB strains

LAB strains	***L. monocytogenes*** **ATCC 7644**		***L. ivanovii*** **ATCC 19119**		***E. coli*** **ATCC 25922**		***S.* Typhimurium ATCC 14028**	
	cells	**CFS**	**cells**	**CFS**	**cells**	**CFS**	**cells**	**CFS**
Ent. faecium MI201	+++	++	+++	++	++	+	+++	+
L. brevis MI202	+++	++	+++	++	+	+	+++	+
L. *farciminis* MI203	+++	+	+++	+	+++	+	+++	+
L. fermentum MI204	+++	+	+++	+	+++	+	+++	+
Pediococcus pentosaceus MI205	++	+++	++	+++	+++	+	+++	+
Weissella cibaria MI206	+++	++	+++	++	+++	+	+++	+
L. plantarum MI207	+++	+	+++	+	+++	+	+++	+
L. plantarum MI208	+++	+	+++	+	+++	+	+++	+
P. acidilactici MI209	++	+	++	+	+++	+	+++	+
P. pentosaceus MI210	+++	+	++	+	+++	+	+++	+
P. pentosaceus MI211	++	+	++	+	+++	+	+++	+
L. brevis MI212	+++	+	++	+	+++	+	+++	+
P. pentosaceus MI213	++	+	++	+	++	+	++	+
P. pentosaceus MI214	+++	+	+++	+	++	+	+++	+
P. acidilactici MI215	+++	+	+++	+	+	+	+++	+

*(-) inhibitory activity absent; (+) inhibition halo of 1-5 mm diameter; (++) inhibition halo of 5-10 mm diameter; (+++) inhibition halo of >10 mm diameter.

Diguță et al. (2020) observed that some *Pediococcus* strains exhibited low antifungal activity against *A. flavus* and *A. niger*. Conversely, five strains of *P. pentosaceus* isolated by Ouili et al. (2023) demonstrated a high ability to suppress the growth of *A. flavus*. The *L. fermentum* MI204, *L. plantarum* MI207, *P. pentosaceus* MI210, and *P. pentosaceus* MI211 strains exhibited pronounced antifungal activity against *B. cinerea*.

Table 3. Antifungal activity of the LAB strains

Bacterii lactice	***A. brasiliensis***	*A. carbonarius*	***A. flavus***	***A. niger***	***A. ochraceus***	***B. cinerea***	*F. oxysporum*	***F. proliferatum***	*P. digitatum*	*P. expansum*
Ent. faecium MI201	-	+	-	-	-	-	-	-	+++	+
L. brevis MI202	+	-	-	-	-	-	+	-	+++	+
L. farciminis MI203	-	+	-	-	-	-	++	+++	+++	+
L. fermentum MI204	+	+	-	-	-	+++	++	+++	+++	+
P. pentosaceus MI205	-	-	-	-	-	-	+	-	-	-
W. cibaria MI206	-	-	-	-	-	-	+	-	-	+
L. plantarum MI207	-	-	-	-	-	+++	+	+++	+	+
L. plantarum MI208	-	-	-	-	-	-	++	+++	++	++
P. acidilactici MI209	-	-	-	-	-	-	-	+	-	-
P. pentosaceus MI210	-	-	-	-	-	+++	++	+	+	+
P. pentosaceus MI211	-	++	-	-	-	++	-	+++	+	-
L. brevis MI212	-	+++	-	-	-	-	+++	+++	+++	+++
P. pentosaceus MI213	-	-	-	-	-	-	-	-	-	+
P. pentosaceus MI214	-	-	-	-	-	-	-	-	-	-
P. acidilactici MI215	-	-	-	-	-	-	-	-	-	-

*(-) inhibitory activity absent; (+) inhibition halo of 1-5 mm diameter; (++) inhibition halo of 5-10 mm diameter; (+++) inhibition halo of >10 mm diameter

A spectrum of activity was observed against *F. oxysporum* and *F. proliferatum*, with the *L. brevis* MI212 strains demonstrating a notable antimicrobial capacity against both *Fusarium* species (Table 3). The effectiveness of LAB strains against *Penicillium* sp. was demonstrated to vary considerably. *Ent. faecium* MI201, *L. brevis* (MI202 and MI212), *L. farciminis* MI203, and *L. fermentum* MI204 demonstrated a notable capacity to inhibit the

growth of *P. digitatum*, while *L. brevis* MI212 exhibited a similar effect on *P. expansum*, as shown in Table 3. The results presented here are consistent with those previously published by Diguță et al. (2020).
Matei et al. (2017) revealed that the Kombucha consortium (or SCOBY which stands for symbiotic acetic/lactic bacteria and yeast) exhibited the most significant inhibition against *B. cinerea*, less significant inhibition against *P. expansum,* and no inhibition against *A. carbonarius* and *A. flavus*.

2. Bacteriocin and EPS production

Lactic acid bacteria are known to produce bacteriocins, which have been demonstrated to possess significant bioactive antimicrobial properties (Daba & Elkhateeb, 2020; Islam et al., 2020; Daba et al., 2022; Bhattacharya et al., 2022; Pristavu et al., 2022; Perez et al., 2022; Kumar et al., 2023). These bacteriocins could potentially be utilised as a natural preservative and may represent a promising alternative to antibiotics, given their efficacy in controlling foodborne pathogens (Daba & Elkhateeb, 2020; Bhattacharya et al., 2022; Perez et al., 2022). The LAB strains that demonstrated the ability to inhibit the growth of the susceptible strain of *S. thermophilus* ATCC 19258 are considered producers of bacteriocins or other active compound(s). A summary of the data on the antimicrobial activity of the crude bacteriocins present in cell-free culture supernatants of LAB strains against *S. thermophilus* ATCC 19258 is presented in Table 4.
The results demonstrated that *L. fermentum* MI204, *L. brevis* MI212, *P. pentosaceus* (MI211, MI213, and MI214) and *P. acidilactici* (MI 209 and MI215) strains exhibited the greatest inhibition zones surrounding the inoculation spots. Additionally, *Ent. faecium* MI201, *L. brevis* MI202, and *P. pentosaceus* MI205 strains can produce some active compounds with inhibitory activity against the tester strain. In a study conducted by Hwanhlem et al. (2014), *Lactococcus lactis* subsp. *lactis* 4KT2W2L and *Ent. faecalis* (KT2W2G, TS9S17, and TS9S19) strains were identified as producers of bacteriocin-like inhibitory substances against some bacteria as indicators, using the agar well diffusion assay. In a relatively published study, Matei et al. (2018) identified three strains of *P. pentosaceus* isolated from the Kombucha consortium as bacteriocin producers.
Moreover, EPS produced by certain genera of LAB have attracted considerable interest due to their ability to extend the shelf life of products, enhance the functional properties of dairy and food products, and promote health (Daba et al., 2021; Korcz, & Varga, 2021; Jurášková et al., 2022).

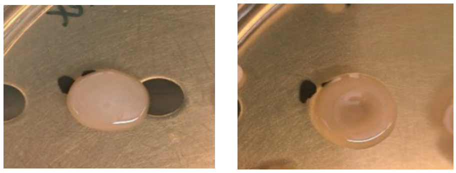

Figure 1. Appearance of colonies producing exopolysaccharides (own source)

Figure 1 illustrates the characteristics of the EPS-producing LAB strains, which were observed to be white, mucoid, and translucent colonies on MRS agar supplemented with 30% sucrose.

Table 4. Bacteriocin and EPS production by LAB strains

LAB strains	Inhibition zone*	EPS production**
Ent. faecium MI201	+	-
L. brevis MI202	+	-
L. farciminis MI203	-	-
L. fermentum MI204	++	+
P. pentosaceus MI205	+	+
W. cibaria MI206	-	-
L. plantarum MI207	-	+
L. plantarum MI208	-	-
P. acidilactici MI209	++	-
P. pentosaceus MI210	-	-
P. pentosaceus MI211	+++	-
L. brevis MI212	++	-
P. pentosaceus MI213	+++	-
P. pentosaceus MI214	+++	-
P. acidilactici MI215	+++	-

*(-) inhibitory activity absent; (+) inhibition halo of 1-5 mm diameter; (++) inhibition halo of 5-10 mm diameter; (+++) inhibition halo of >10 mm diameter
**(-) absent activity; (+) present activity

Screening results revealed that the strains *L. fermentum* MI204, *P. pentosaceus* MI205, and *L. plantarum* MI207 exhibited the potential to produce exopolysaccharides (Table 4). In another study, Ma'unatin et al. (2020) demonstrated that *Fructobacillus fructosus* N4 and *Leuconostoc mesenteroides* (N5, N7, N9, N10) strains were capable of producing extracellular polymeric substances (EPS) when cultivated on MRS agar with a 30% sucrose supplement. Álvarez et al. (2021) showed how a *Lactobacillus plantarum* strain integrated in an edible EPS-based coating from *Weissella confusa* improved the microbiological and physicochemical quality of cherry tomatoes. According to reports, a significant component of the biofilms' resistance mechanism is the EPS matrix.

3. Organic acids production

The available literature suggests that the preservation of LAB is the consequence not of the action of a single compound but rather of the combined effect of metabolites produced by these microorganisms during fermentation.

In the present study, lactic and acetic acids in the CFS of LAB strains were ascertained through using two distinct methods such as plate screening on specific media and HPLC assay. The results from these investigations are presented in Tables 5 and 6, respectively. LAB strains demonstrated the ability to produce organic acids during fermentation. The formation of the largest halos around the inoculation area indicated the highest potential of these strains for lactic acid production. With the exception of *L. fermentum* MI207 and *P. pentosaceus* MI214, the remaining LAB strains were observed to produce acetic acid on solid medium. Of these, *Ent. faecium* MI201, *L. brevis* MI202, and *L. farciminis* MI203 exhibited the highest activity (Table 5).

Table 5. Detection of acetic and lactic acids production of LAB strains through plate screening

LAB strains	Acetic acid production	Lactic acid production
Ent. faecium MI201	+++	++
L. brevis MI202	+++	++
L. farciminis MI203	+++	+
L. fermentum MI204	++	+++
P. pentosaceus MI205	++	+
W. cibaria MI206	++	+
L. plantarum MI207	-	+++
L. plantarum MI208	++	++
P. acidilactici MI209	++	+++
P. pentosaceus MI210	++	++
P. pentosaceus MI211	++	+
L. brevis MI212	++	++
P. pentosaceus MI213	++	++
P. pentosaceus MI214	-	++
P. acidilactici MI215	++	+

*(-) halo absent; (+) halo of 1-5 mm diameter; (++) halo of 5-10 mm diameter; (+++) halo of >10 mm diameter

On the other hand, all LAB strains were found to be able to produce lactic acid on agar medium (Table 5), with *L. fermentum* MI204 and MI207, and *P. acidilactici* MI209 strains demonstrating the greatest ability.

Table 6. Quantification of lactic and acetic acids content by HPLC assay

LAB strains	Acetic acid mg/ml	Lactic acid mg/ml
Ent. faecium MI201	0.42	14.17
L. brevis MI202	0.53	9.43
L. farciminis MI203	0.92	6.64
L. fermentum MI204	**1.55**	16.43
P. pentosaceus MI205	<LoQ	6.46
W. cibaria MI206	0.80	10.96
L. plantarum MI207	0.60	7.04
L. plantarum MI208	0.22	12.47
P. acidilactici MI209	**1.30**	16.86
P. pentosaceus MI210	0.71	14.93
P. pentosaceus MI211	1.05	**20.02**
L. brevis MI212	0.50	15.17
P. pentosaceus MI213	0.47	**20.41**
P. pentosaceus MI214	<LoD	16.06
P. acidilactici MI215	0.69	**27.00**

<LoQ - limit of quantification; <LoD - detection limit

The quantified level of acetic acid by HPLC exhibits considerably lower values, ranging from 0.22 mg/ml for *L. plantarum* MI208 to 1.55 mg/ml for *L. fermentum* MI204. The concentration of acetic acid was below the limit of detection for *P. pentosaceus* MI214 and below the limit of quantification for *P. pentosaceus* MI205 (Table 6). The lactic acid concentration, the principal organic acid with beneficial effects, exhibited a considerable range from 6.46 mg/ml for *P. pentosaceus* MI205 to 27 mg/ml for *P. acidilactici* MI215 (Table 6).

According to Loubiere et al. (1997), lactic acid exerts its inhibitory effects on metabolic processes and cellular proliferation, possibly due to the synergistic interaction between these compound and other secondary metabolites, including acetic and formic acids.

4. Proteolytic and phytase activities

The potential for LAB strains to produce proteases was evaluated on agar skim milk medium. Of the strains tested, nine demonstrated positive proteolytic activity, with the greatest halo surrounding the *Ent. faecium* MI201, *P. acidilactici* MI209, *L. brevis* MI212, and *P. acidilactici* MI215 strains (Table 7).

These outcomes align with those of previous studies in this area. *L. plantarum* subsp. *plantarum* P15 and *E. faecalis* ZZUPF95 exhibited the greatest protease production abilities, as previously reported by Ma et al. (2022). Additionally, Kouadio et al. (2024) reported the production of protease enzymes by ten *L. plantarum* strains, isolated from traditional fermented dockounou paste.

Table 7. Proteolytic and phytase activity of the LAB strains

LAB strains	Proteolytic activity	Phytase activity
Ent. faecium MI201	+++	+++
L. brevis MI202	++	+++
L. farciminis MI203	-	+++
L. fermentum MI204	-	+++
P. pentosaceus MI205	++	+++
W. cibaria MI206	-	+++
L. plantarum MI207	-	+++
L. plantarum MI208	+	+++
P. acidilactici MI209	+++	+++
P. pentosaceus MI210	++	+++
P. pentosaceus MI211	-	+++
L. brevis MI212	+++	-
P. pentosaceus MI213	-	+++
P. pentosaceus MI214	++	+++
P. acidilactici MI215	+++	+++

*(-) halo absent; (+) halo of 1-5 mm diameter; (++) halo of 5-10 mm diameter; (+++) halo of >10 mm diameter

Previous studies demonstrated that different LAB strains possess the ability to produce intracellular and/or extracellular phytases (Cizeikiene et al., 2015; Bhagat et al., 2020; Sharma et al., 2020).

In the present study, all LAB strains presented extracellular phytase activities that were significantly elevated after 96 hours of incubation, with the sole exception of *L. brevis* MI212.

As reported by Cizeikiene et al. (2015), *P. acidilactici* (KTU05-7) and *P. pentosaceus* KTU05-8 have been identified as the strains with the highest extracellular phytase activity. Also, another strain of *P. acidilactici* SMVDUDB2, isolated from traditional fermented cheese product, has been demonstrated to exhibit extracellular phytase enzyme activity, with an optimum pH of 5.5 and temperature of 37°C, being thermostable at 60°C (Bhagat et al., 2020). Moreover, *L. brevis* strain can synthesise phytase both within and outside their cells (Sümengen et al., 2012).

5. Antioxidant activity

The antioxidant potential of LAB strains was assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. The findings indicated that all fifteen LAB strains demonstrated notable antioxidant activity ranging from 45.79% to 52.58% (Figure 2). Among the strains tested, *P. pentosaceus* MI205 displayed the most pronounced antioxidant efficacy, exhibiting a DPPH activity of 52.58%.

These findings are consistent with those previously reported in the literature (Diguță et al., 2020; Koaudio et al., 2024).

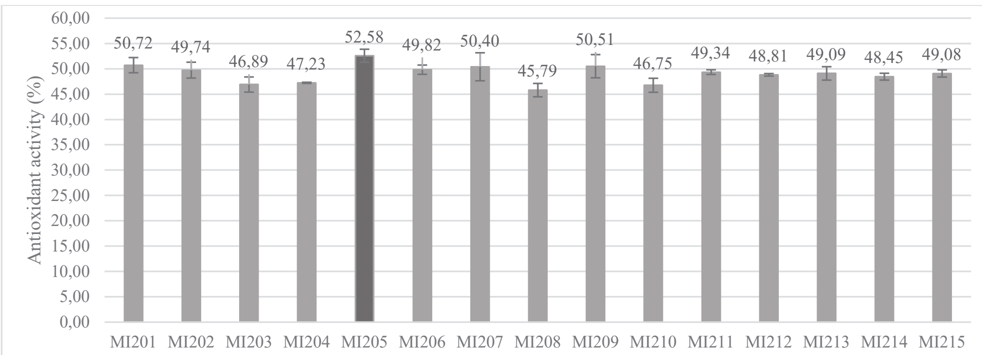

Figure 2. Antioxidant activity (%) of LAB strains

6. Preservation of LAB strains by lyophilization

The freeze-drying (or lyophylization) procedure is frequently employed to preserve and store LAB strains for biotechnological applications, with the advantage of maintaining the native microbial structure and viability of bacterial cells during storage (Meena et al., 2023). However, the process of freezing and subsequent freeze-drying leads to membrane damage and osmolarity stress due to the formation of ice crystals during the freezing step. Thus cryoprotective agents are required to extend the viability of LAB bacteria.

In our study, glucose was employed as a cryoprotective agent to enhance the viability of microorganisms during the freeze-drying procedure and facilitate their future utilization. The viability percentage of freeze-dried LAB strains ranged from 50.68% (*Ent. faecium* MI201) to 98.40% (*L. plantarum* MI207) (Figure 3). There is considerable variability in the efficacy of glucose as a cryoprotectant agent between different LAB strains.

In their study, Diguță et al. (2020) investigated the storage stability of the probiotic *Pediococcus* sp. through freeze-drying techniques utilising two different cryoprotectants: glucose and sucrose. Their findings indicated a high viability rate of 86–92%, which provides evidence of the effectiveness of their methodology. Consequently, further research is required to select the most appropriate cryoprotectant agents for conditioning LAB strains, with the highest viability rates and cost-effectiveness.

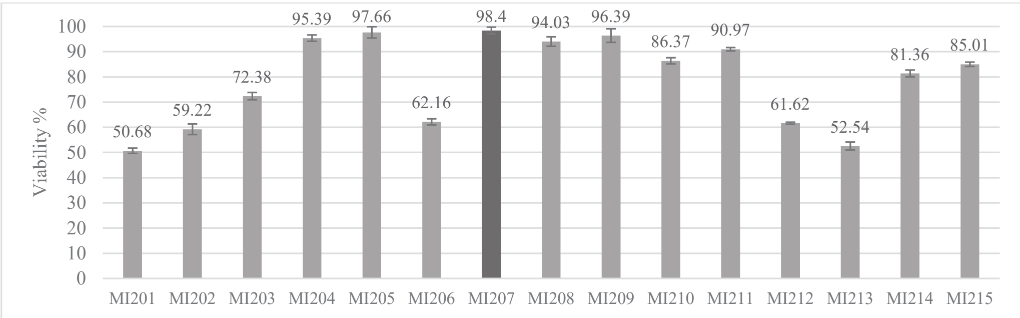

Figure 3. Viability after lyophilization (%) of LAB strains

CONCLUSIONS

The challenge of maintaining the nutritional value and sensory properties of perishable fruits for a longer period while ensuring its suitability for distribution and consumption is significant in terms of technological obstacles. Among the fifteen LAB strains tested, *Lactobacillus plantarum* MI207 demonstrated the greatest efficacy against foodborne pathogens, as well as several other beneficial abilities due to their probiotic properties. In addition, the EPS production is encouraging for testing the positive LABs for the fruit coating potential. Nevertheless, there is still much to be researched regarding the potential of *Lactobacillus plantarum* MI207 as a sustainable candidate for enhancing the shelf-life of fresh and minimally processed foods.

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CURRENT STATE OF RESEARCH ON THE ECONOMIC AND SOCIAL IMPORTANCE OF TRADITIONAL PRODUCTS IN THE CONTEXT OF FOOD PRODUCTION GLOBALIZATION

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Abstract

In the context of food globalization, traditional products play a special role in preserving cultural identity and supporting the local economy. This research aims to examine the economic and social importance of these products, using data collection methods such as specific laboratory analyses, as well as surveys, and interviews with producers and consumers. The study seeks to highlight the differences between traditional and industrial products, as well as to understand the significance of the concept of traditional products for each consumer and how these differ from industrial products, focusing on the perceived benefits and challenges associated with each type. The results show that, although traditional products are perceived as healthier and more authentic, they face difficulties in competing with industrial products. The paper provides recommendations for policies that support the preservation and promotion of traditional products.

Key words: *traditional products, industrial products, globalization.*

INTRODUCTION

The globalization of food production has transformed markets and agricultural practices worldwide, bringing both challenges and opportunities for traditional products.
This research aims to explore the economic and social importance of traditional products in the context of globalization, providing a detailed analysis of their dynamics within the global economy. We will assess current trends, challenges, and opportunities, and identify ways in which traditional products can be protected and promoted.

MATERIALS AND METHODS

In order to write this paper, a detailed study of various published works was carried out on topics like globalization, food production, traditional products and customers perception of the traditional products. Scientific articles and papers were selected from Science Direct, PubMed, Google Scholar, Web of Science databases using multiple search keywords related to traditional foods market, food globalization, or European Union policies. Eligible articles were selected on this topics and more. The selected articles were analyzed for their eligibility and to extract the data necessary for the creation of this paper.

RESULTS AND DISCUSSIONS

The Economic and Social Impact of Traditional Products

To determine the economic and social impact of traditional products, this research seeks to answer two fundamental questions:
1. What does a traditional food or product mean to the Romanian consumer?
2. What is the real difference between traditional and industrial products?
In the current context of rapidly changing consumer preferences, understanding the significance of the concept of traditional products for each individual consumer, and distinguishing them from industrial products, is important for economic, social, and cultural reasons. By exploring the question, "What does

a traditional food or product mean to the Romanian consumer?", we aim to understand the perceptions and values that consumers associate with these products. This understanding can help producers develop more effective strategies to meet the needs and desires of consumers. Additionally, this can support efforts to preserve Romanian culinary heritage, contributing to the preservation and promotion of local culture in the face of globalization.

By addressing the question, "What is the real difference between traditional and industrial products?", the study intends to provide a fundamental knowledge base to guide informed choices and educate consumers. Through a better understanding of the Romanian consumer, more well-founded strategies can be developed for preserving culinary heritage, fostering local economic development, and enhancing consumer health and well-being. Furthermore, the study will support policymakers and industry stakeholders in formulating policies and practices that protect and promote traditional products in a globalized market (Stanciu et al., 2022).

Definitions and Context

Globalization (as defined in the Romanian Dictionary - Dex): "the phenomenon of transforming the world into a unit, which manifests on a global scale through specific means" (https://dexonline.ro/).

Globalization (as defined by Wikipedia): "the modern term used to describe the changes in societies and the global economy, resulting from significantly increased international trade and cultural exchanges. It describes the growth of trade and investments due to the reduction of barriers and the interdependence between states" (https://ro.wikipedia.org/wiki/Globalizare).

Tradition (as defined in the Romanian Dictionary - Dex): "A set of conceptions, customs, practices, and beliefs that historically become established within social or national groups and are transmitted (orally) from generation to generation, constituting the specific trait of each social group. Custom, practice, habit, rule, order, usage" (https://dexonline.ro/).

Traditional product (as defined by MARD): "A food product manufactured on the national territory, using local raw materials; which does not contain food additives; which follows a traditional recipe, a traditional production and/or processing method, and a traditional technological process, and which is distinguished from other similar products in the same category" (https://infocons.ro/produse-traditionale-romanesti).

The Role of Traditional Products

To what extent is our eating behavior biologically determined, and to what extent are our choices based on cultural reasons? Some peoples develop extremely varied preferences regarding certain foods, such as coffee, sweets, or certain spices, which they integrate so deeply into their daily lives that they become true community rituals, and eventually become representative of the local culture. This is why the English and the French eat differently and have been teasing each other on culinary topics for centuries. The English call the French "frogs", and the French respond by saying that "the English have a hundred religions but only one sauce". Pizza is another world phenomenal food, which quickly went from complete obscurity to being Americans' favorite dish. Our nutritional needs and some very general preferences are strictly biologically determined. The environment can modify our needs to a certain extent, but it cannot completely change the biological foundation - we need proteins or vitamin C, regardless of what we believe or think. Preferences, however, are strongly influenced by culture and social influences (Vintilă & Iancu, 2009). "Convinced that their health is in their own hands, the modern consumer has become much more attentive and selective regarding their diet" (Ossipow, 2022). In the past, people's diets were heavily limited to what they could gather or produce at home. However, as technology and society evolved, distances between communities became easier to cover, and access to an increasingly diverse range of food became more convenient (Brandabur & Tănase, 2010). In our times, globalization has revolutionized the trade, production, and distribution of food products, with both positive and negative effects. Yet, at the root of the purchasing decision lies the individual, with their options and preferences. What we aim to explore are precisely these preferences, which have always been influenced by cultural, social, or environmental factors (Săgeată et al., 2014).

The Nutritional Value of Traditional Products

The modern consumer, beyond the nutritional and health attributes of food, expects products to stand out through sensory qualities, natural ingredients, and high quality. Therefore, this results in an increased interest in traditional products. Convinced that their health is in their own hands, the modern individual has become much more attentive and selective about their diet, as noted by (Ossipow, 2022). In a study conducted in Romania, Reget et al. (2020) compared traditional products (smoked and dry-cured ham, smoked ham, and sausages) collected from a traditional production unit in Sălaj County with similar industrial products purchased from hypermarkets. The traditional smoked dry-cured ham has a higher protein content (28.54%) and lower water content (55.23%) compared to industrial products. Traditional sausages have a higher fat content (32.25%) and a greater collagen-to-protein ratio, but do not exceed legal limits. Traditional products are of superior quality compared to similar Swiss or German products regarding water content. Industrial smoked dry-cured ham presents a significantly higher water content and lower protein content, reducing its nutritional value. In smoked ham, the differences in water and protein content are significant, with the traditional product being nutritionally superior. Industrial sausages have a much higher fat content (45.14%) and lower protein content (16.84%) compared to traditional products. The study revealed significant differences between pork products obtained traditionally and those produced industrially. Traditional products have a higher biological value due to their increased protein, fat, and collagen content, and reduced water content. Histological analysis highlighted cellular hyperhydration and increased fibrous tissue in industrial products, confirming their inferior quality compared to traditional products. Traditional cured meats offer a superior nutritional profile compared to industrial ones, with fewer additives and controlled levels of preservatives, making them a healthier choice for consumers, according to a similar comparative study conducted by (Halagarda et al., 2018) compared the nutritional value and potential chemical risks of traditional and conventional cured meats from Poland, obtaining the following results:

Tabel 1. Comparative nutritional indicators for traditional and industrial meats (Halagarda et al., 2018)

Nutritional Indicator/ Food Safety	Traditional sausages	Industrial sausages
Proteins	High content	Low content
Zinc	High content	Low content
Magnezium	High content	Low content
Potassium	High content	Low content
Calcium	High content	Low content
Total Ash	Low content	High content
Water/Protein Ratio	Lower ratio	Higher ratio
Polyphosphates	Absent	Present
Nitrites	Low quantities	Higher quantities
Nitrates	Moderate	High content
Total and Added Phosphorus	Moderate	High content
Cadmium	Moderate	High content
Lead	Moderate	High content

Functional foods

Are also closely linked to traditional products through the use of plants for their functional benefits, the recognition of the importance of culinary and medicinal traditions, and the understanding of the ecological and evolutionary role of plants in nutrition. Modern research in this field provides scientific validation for this traditional knowledge, highlighting the importance of integrating both traditional and scientific approaches in promoting health and well-being. Many traditional products, such as kimchi, kefir, and green tea, are now recognized for their functional properties. Modern research confirms the benefits of these foods, highlighting their bioactive compounds and mechanisms of action (Marriott, 2000).

Traditional Products and the European Union

Traditional food products play an important role in supporting local economies, contributing to the development of rural communities, creating jobs, and preserving cultural heritage. The EU's geographical indications system protects the names of products that come from specific regions and possess certain qualities or whose reputation is linked to the geographical area where they are produced. The distinction between PDO (Protected Designation of Origin) and PGI (Protected Geographical Indication) primarily concerns the minimum limits of raw materials from the region of origin and the extent to which the production activity takes place in a specific region. The geographical indication (GI) is used solely for flavored wines and alcoholic beverages. The European quality label ensures and promotes the origins of a product, local traditions, and the specific characteristics of many European products with unique features (Rotari, 2015). Romania has 13 products protected within the European Union. Traditional products protected by the EU's quality schemes, such as geographical indications (GI) and traditional specialties guaranteed (TSG), represent food and beverages that benefit from protection and recognition at the European level due to their unique characteristics, linked to geographical origin or traditional production methods (https://www.euipo.europa.eu/ro/gi-hub).

Geographical Indications (GI) are a protection system for products that have a specific connection to a particular geographical area. These products must meet certain criteria and be produced in a specific region, using traditional knowledge and methods. GIs are divided into two categories:

PDO (Protected Designation of Origin): This term is used for products that are produced, processed, and prepared in a specific geographical region and that possess qualities or characteristics primarily attributed to that geographical environment and traditional production methods. Romania has one certified PDO product: Telemeaua de Ibănești.

PGI (Protected Geographical Indication): This term applies to products that have a connection to a specific region, but not all stages of production must take place in that area. It is sufficient for one of the stages of production, processing, or preparation to occur in the respective region. Romania has 11 certified PGI products: Salinate de Turda, Pită de Pecica, Plăcinta Dobrogeană, Telemeaua de Sibiu, Cârnații de Pleșcoi, Scrumbia de Dunăre afumată, Novacul afumat din Țara Bârsei, Salamul de Sibiu, Magiunul de prune de Topoloveni, Cașcavalul de Săveni, Salata cu icre de știucă de Tulcea.

Traditional Specialties Guaranteed (TSG): TSG refers to products that have a traditional composition or production method, without necessarily being linked to a specific geographical area. These products must reflect a tradition of at least 30 years and be recognized for their traditional character. Romania has one certified TSG product: Salată tradițională cu icre de crap (traditional carp roe salad). The EU's quality schemes for protected traditional products play a crucial role in safeguarding and promoting the diversity and authenticity of European food products, while also providing significant economic and cultural benefits (https://romania.representation.ec.europa.eu/indicatii-geografice-si-sisteme-de-calitate-produse-romanesti-protejate-ue_ro).

These schemes protect product names from unauthorized use and imitation, ensuring that only authentic products that meet the defined specifications can use the protected designation. Quality schemes contribute to the recognition and promotion of traditional products, encouraging the maintenance of high-quality and authentic standards. Products protected by these schemes often command a premium price on the market due to the recognition of their quality and authenticity, which can bring additional economic benefits to local producers. These schemes help preserve traditional production methods and cultural heritage, contributing to the preservation of regional identity and biodiversity.

The European Community Strategy on Traditional Products

The development of traditional food production constitutes an EU community strategy, regulated by strict production and sales criteria. The European marking system for traditional foods is based on assigning distinctive signs related to origin and quality, ensuring and protecting over 1,000 registered names. In 2015, a total of 1,317 traditional food products were registered in Europe, with the majority being Protected Geographical Indication (PGI) and Protected Designation of Origin (PDO) products. The distribution of traditional agri-food production across European countries varies, with Italy leading with 21.4% of registered products, followed by France and Spain. The Gini-Struck and Herfindahl-Hirschman concentration indices for traditional products in the EU do not indicate an excessively concentrated market, with values below alert thresholds.

Romania, despite its rich history and cultural heritage, does not fully exploit the niche of traditional products. To compete with large producers and retailers in the EU, the domestic food sector could adopt strategies linking traditional production with tourism or specialized food stores. Strict regulations have reduced the number of certified products. As of December 2015, 513 products were registered, the majority being meat products, dairy, fruits and vegetables, and bakery products. Between 2012 and 2015, the number of traditional producers significantly decreased, with most concentrated in the counties of Brașov, Argeș, and Maramureș (Stanciu, 2015). Traditional products present considerable potential in both local and international markets. They serve as a means of diversifying the food supply while simultaneously preserving local identity, traditions, and the unique characteristics of communities. At the European level, measures taken by officials in recent years have led to a significant increase in the registration of traditional products under the PDO, PGI, or TSG labels. This suggests that food traditions could represent a strategic advantage for Romanian production in a highly competitive national and European market, which remains underutilized by local producers (Bichescu & Stanciu, 2017). As points of Mirea & Casangiu Siea, (2022), gastronomic tourism, being connected to local communities and their customs, inevitably becomes a promoter of sustainability, thus evolving into a form of sustainable tourism. Gastronomic tourism fully contributes to the promotion and development of local traditional food products, representing a generator of the multiplier effect of tourism (Soare, 2018). These aspects are advantages for the local community. Consumers are motivated to buy organic food due to positive perceptions related to health, taste, and the environment (Nan et al., 2019). These perceptions are supported by the belief that organic products are more natural and less processed compared to conventional foods. This suggests that the market for traditional products may benefit from increased demand due to consumer trends towards seeking more natural and authentic products (Bichescu & Stanciu, 2017). Local production and the tourism industry are interdependent, contributing to both economic development and cultural preservation. The development of tourism in rural areas must be sustainable, protecting natural resources and cultural traditions. Artisanal production plays a crucial role in preserving local identity and attracting tourists, having a significant economic impact on rural communities (Haller, 2011).

The Contribution of Traditional Products to the European Economy

In 2017, the sales value of products under the GI/TSG schemes reached EUR 77.1 billion, representing 7% of the total EU food and beverage sector. Agricultural and food products under the GI schemes accounted for 35% of this value, highlighting their importance in the agricultural and food sectors. Total exports of GI/TSG products were estimated at EUR 32.1 billion, representing 42% of the total sales value. Of this, 22% was destined for non-EU markets, while 20% went to intra-EU markets. These significant exports contribute to generating income for local producers and boosting the local economy by expanding market access.

Value Premium

GI/TSG products had an average value premium of 2.07 in 2017, meaning that the sales value of GI products was 2.07 times higher than that of similar products without a GI/TSG label. This value premium indicates that protected traditional products are perceived as higher quality, allowing for higher prices and increased income for local producers. The implementation of GI/TSG schemes involves an increased number of procedures and controls, which improve quality management along the supply chain. This aspect helps ensure product quality, attracting a segment of consumers willing to pay a premium price for authentic and high-quality products (Tanasa et al., 2015).

GI and TSG cover a wide range of products, from wines and spirits to cheeses and processed meat products. For example, in 2017, wines accounted for 51% of total sales value, followed by agricultural and food products (35%) and spirits (13%). The diversity of these products supports a variety of local sectors, from agriculture to the food industry, strengthening the local economy by creating jobs and stimulating local production. Registering and protecting products under the GI/TSG schemes contributes to the preservation of local heritage, genetic resources, landscapes, and cultural traditions. This not only supports the sustainable development of local communities but also attracts tourists and investors interested in the region's authentic and traditional products. (European Commission, 2021).

Traditional products can also contribute to the sustainable economic development of communities, offering employment opportunities and generating income. This can lead to a reduction in migration to urban areas and help maintain a demographic balance in rural regions. Economic development based on community products can be sustainable from an ecological perspective as well, since these products are often made using traditional practices that respect the environment. (Buzoianu et al., 2023)

Traditional products play an important role in maintaining social cohesion and shaping community identity. By promoting social interactions and cultural events, they strengthen the bonds between community members. Additionally, they are essential for reaffirming cultural identity in a homogenized world, while also contributing to the sustainable economic development of communities (Belo Moreira, 2004). Thus, traditional products represent more than just a source of food for the community; they are a way to preserve cultural heritage and a means to ensure the long-term cohesion and prosperity of the community (Necula et al., 2022).

The Beneficiaries of Traditional Products

tudies on the consumer niches that prefer traditional products have yielded quite varied results. For example, in a study conducted by Gundala & Singh (2021), surveyed 770 individuals from the United States and identified the following demographic differences in actual purchasing behavior: Regarding gender, there were no significant differences between men and women in purchasing behavior. Regarding age, the 41-50 age group was more likely to purchase organic foods. In terms of income, higher income was associated with a greater likelihood of buying organic foods. Regarding education, higher levels of education were correlated with a greater probability of purchasing organic foods.

In a study conducted by (Turek Rahoveanu et al., 2008) the following consumer niches for traditional products were identified in Romania: the profile of the traditional food consumer is defined as female, living in urban areas, with an income between 1,000 and 2,000 lei, generally students or professionals such as engineers, teachers, doctors, and economists. Individuals from rural areas, especially men over the age of 56 with incomes below 1,000 lei, and occupations such as administrators, farmers, company owners, self-employed professionals, or unemployed, do not consume traditional products.

The market for traditional agri-food products is primarily segmented by residence and age, with less influence from income and occupational status (Chistruga & Crudu, 2015). Urban populations are more open to trying new products, have higher incomes, and are more receptive to purchasing value-added products and those with higher levels of processing compared to rural areas.

The demand for traditional food products varies by age group, with preferences such as bread and bakery products for those under 25, meat products for those aged 41 to 55, and dairy products for individuals over 56. Globalization is a complex system or phenomenon, sometimes even contradictory, which has been viewed and analyzed from a multitude of perspectives by those who have taken on this responsibility. The globalized or economic-centric person risks becoming a dehumanized individual who lives only for production and consumption, devoid of culture, politics, meaning, conscience, religion, and any form of transcendence (Felea, 2023).

Community identity is closely linked to the traditional products specific to each community, which are often considered symbols of local distinctiveness. These products reflect the history, culture, and natural environment of a community, contributing to the creation of an identity (Komer & Cop, 2022). They are the result of knowledge and practices passed down from generation to generation, many becoming family legacies and an integral part of both the personal cultural heritage of each individual and that of the community. By promoting and protecting traditional products, communities can reaffirm and strengthen their identity in an increasingly globalized world. This process of identity reaffirmation can be relevant in combating the negative effects of globalization, such as the loss of cultural diversity and local identity. Moreover, a strong community identity can generate a sense of pride and belonging, which motivates community members to actively engage in the protection and promotion of their cultural heritage (Belo Moreira, 2004).

Challenges and Opportunities in the Context of Globalization

Globalization has brought with it a demand for standardization, which can lead to the uniformity of products and processes. This poses a challenge for producers of traditional products, who often rely on unique characteristics achieved through culturally specific and specialized production methods. Standardization requires changes in production processes to comply with international regulations, which can impact the quality and authenticity of these products (Bercu et al., 2010).

Traditional products typically involve manual or semi-manual production methods, making them more expensive compared to mass-produced goods. This puts them at a disadvantage in the global market, where price is often the decisive factor for consumers (Rahoveanu et al., 2008). Traditional products are often tied to the cultural heritage and identity of a community, but to be attractive in global markets, these products may be altered to conform to international tastes and preferences. This can lead to a dilution of their cultural significance (Boldea & Buda, 2014). Another risk is that traditional products may be copied and mass-produced by entities with no connection to the original communities, without respecting intellectual property rights and without compensating the communities for the use of their traditional knowledge and techniques (Drori, 2006).

The close interconnection of communities also generates increased risks to food security, which is crucial for human survival and is directly affected by conditions of poverty and inequality. Conflicts, climate change, rising prices, and economic shocks (including the pandemic) are key factors that have triggered major food crises. The UN has set a goal to eradicate hunger and ensure global food security by 2030. To address these crises and improve food security, global cooperation and effective policies are necessary. Governments must invest in climate-resilient agriculture, improve resource management, and promote alternative energy sources. Additionally, the use of technology to provide information to farmers and the application of the principles of reduce, reuse, and recycle can help reduce the negative environmental impact and improve food security both globally and for local communities (Miu & Mihailescu, 2023)

Standardization is one of the main effects of globalization that impacts traditional products. The globalization process involves increasing pressure for the harmonization of standards and procedures, which can lead to the uniformity of products. International standards, such as ISO, while useful for facilitating international trade and ensuring quality, may impose strict rules that do not take into account the local specificities of traditional products. For example, the ISO 9000 standards were developed to ensure uniform quality management, but they can be criticized for not always reflecting the local particularities and values associated with traditional products. (Buzoianu et al., 2023).

Competition: Globalization increases competition in both local and international markets, exposing traditional products to competition with industrial products, often produced by large multinational corporations. These corporations benefit from economies of scale, advanced technology, and global distribution networks, allowing them to offer products at lower prices and with greater availability than traditional products (Scoppola, 2021). In the agri-food sector, for example, multinationals like Nestlé and Unilever have the capacity to take over local markets through strategic acquisitions and vertical integration of production, which can marginalize traditional local producers.

Loss of Identity: Another critical aspect of globalization is the loss of the cultural identity of traditional products. As local products are adapted to meet international standards and compete in global markets, they may lose the

unique characteristics that set them apart. This process, often referred to as "cultural homogenization", can lead to a significant loss of cultural diversity. In the fields of agriculture and food, this phenomenon is evident in the way local products are standardized and marketed under global brands, thereby diluting their authenticity and specificity (Buzoianu et al., 2023; Belo Moreira, 2004).
In addition to these three major challenges, globalization brings other difficulties:
Financial Instability: The penetration of fluctuating foreign capital can create economic instability and vulnerabilities in traditional sectors.Pollution and Environmental Issues: Rapid industrial development and urbanization can lead to environmental degradation, affecting sustainable traditional practices and disrupting the local ecological balance (Buzoianu et al., 2023).
Exposure to Global Crises: COVID-19: The opening of markets to global flows exposes local communities to external factors that can disrupt and even threaten their well-being. Globalization has made national food systems vulnerable to external shocks, such as price fluctuations on international markets and disruptions to global supply chains. The COVID-19 pandemic significantly disrupted supply chains for agricultural inputs (seeds, plant protection products) and food products, affecting both primary supply as well as food processing and distribution. International transportation was delayed, and restrictions led to difficulties in sourcing essential inputs for agriculture. The economic crisis associated with the pandemic reduced the purchasing power of the population, impacting demand for higher-quality products, such as organic or traditional foods. The pandemic exacerbated poverty in rural areas, disproportionately affecting small farmers and agricultural workers who lost income due to restrictions and the closure of farmers' markets (Alexandri et al., 2020)
Globalization presents challenges, but the opening of markets can also bring new opportunities for development and expansion. It is essential to find a balance that protects and promotes the cultural identity and specificity of these products. Solutions could include policies that support local producers, the promotion of origin labeling, and educating consumers about the value of traditional products (Schileru, 2005). These strategies could help preserve cultural heritage and ensure long-term economic sustainability for local communities.

CONCLUSIONS

The Romanian market for traditional products is still in a development phase, with Romanian consumers positioned between two extremes: accessible but ultra-processed industrial products and those perceived as traditional but often financially inaccessible. For the Romanian consumer, there is a discrepancy between the official definition of a traditional product and their personal perception of what traditional means. After liberation from the communist regime, the transition to capitalism, and integration into the European Union, Romanian society has undergone numerous changes, the effects of which are also visible in today's eating habits. In a world where McDonaldization coexists with Protected Geographical Indication products, Romanian consumers strive to navigate their options and understand the true meaning of the term "Traditional Product", as well as the essential differences between traditional and industrial products.

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QUALITY ASSESSMENT OF SOME ASSORTMENTS OF CHICKEN HOT DOG SAUSAGES

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Abstract

The purpose of this paper was the comparative assessment of the quality of some assortments of chicken hot dog sausages sold in Romania. Two batches of five varieties of hot dog sausages were taken in the study: Caroli, Fox, Cris-Tim, Meda and Pikok/Lidl brand, coded from A to E. Sensory (five-point scale method) and physico-chemical properties were analyzed. Were determined the pH of the products, the content of mineral substances (by calcination at 550°C), the content of water, lipids, proteins, collagen and salt (with the Food-Check infrared spectrophotometer). The results showed very high differences between products in terms of fat content (between 13.5% and 25.1%), the variability was lower for proteins (between 16.6% and 19.3%) and water content (between 55.83% and 66.94%). The salt content had the highest value of 2.83%, exceeding the maximum standard limit, only in the case of C product. The results of the sensory analysis revealed a minimum score for product E (10.63 points/"unsatisfactory product" according to quality standards), compared to product B which obtained the best score among all the analyzed assortments (17.70 points/ "good product").

Key words: *chicken hot dog sausages, quality.*

INTRODUCTION

Recently, the convenience food has become very popular in the market (Contini et al., 2020). At the same time, there is a trend to reduce the consumption of meat and animal products, and consumers are looking more recently the vegan products that they can use in the traditional way they are familiar with (Rybicka et al., 2024). Such products also include plant-based sausages, which can be used both as part of the main course (a hot dog) and as a snack (Kowalczewski et al., 2024).

Nowadays, the growing understanding of the relationship between diet and food ingredients, and its effect on health (Dos Santos, 2020a), has moved consumers to become more conscious and looking for healthier processed foods (de Carvalho et al., 2020). Therefore, many researchers focused on new approaches to develop meat products with better nutritional characteristics; one of the most employed strategies is the reduction of animal fat, which has an elevated saturated fat level More suitable ingredients, such as dietary fibers and edible oils, have been used as a fat substitute (Câmara et al., 2020; de Carvalho et al., 2020; Felisberto et al., 2015).

Marine and vegetable oils are rich in PUFAs content, and their use has been recommended in a healthy diet (Heck et al., 2019). Usually, oils are incorporated into meat products through pre-emulsions, and, more recently, emulsion gels (EGs) as a more suitable strategy to improve nutritional and technological aspects of meat products (Paglarini et al., 2019).

Dietary fibers have health claims, and important technological properties, such as water/oil holding ability, stabilizer, thickener, and gelling properties (Biswas et al., 2011) to elaborate EGs with suitable qualities (dos Santos et al., 2020). Among the proteins that can be used as a structuring agent to create soft-solid EGs, collagen presents suitable functional properties due to its emulsifying and high gelling properties (Gómez-Guillén et al., 2011).

Besides, some by-products of the meat industry are rich in collagen content, such as pork skin that due the extender and binder characteristics has been used to enhanced meat product quality (de Oliveira Fagundes et al., 2017). The purpose of this paper was the comparative assessment of the quality of some assortments of chicken hot dog sausages sold in Romania.

MATERIALS AND METHODS

In order to characterize the quality of some assortments of chicken hot dog sausages, two batches of five varieties of chicken hot dog were taken in the study: Caroli, Fox, Cris-Tim, Meda and Pikok/Lidl brand, randomly coded from A, B, C, D and E (ten samples/five products of two batches from different manufacturer).

Sensory characteristics were analyzed by tasting, using the scoring method;

The samples were minced preliminarily finely ground and homogenized before analysis using an electric shredder (according to the five different manufacturers). Subsequently, the amounts required by each method were used to run 10 analytical replicates per trait.

The water, proteins and lipids contents were assessed on the Omega Bruins Food-Check Near InfraRed (NIR) spectrophotometer (Bruins Instruments GmbH, Germany); the crude ash content was assessed by furnace muffle calcination in a Nabertherm B180 device (Nabertherm GmbH, Germany) (550°C for 24 h after a preliminary carbonization on Bunsen burner flame) (AOAC, 2000; AOAC, 2005).

The nitrogen-free extract (NFE) was calculated by difference, using the Equation (1).

$$NFE\ (g/100\ g) = 100 - Water - Ash - Proteins - Lipids \quad (1)$$

The gross energy value was calculated via the Atwater Equation (2), which uses the caloric value of each organic matter compound in the analysed matrix (total proteins, lipids, nitrogen-free extract - NFE) (FAO, 2003).

$$GE\ (kcal/100\ g\ meat) = g\ proteins \times 4.27\ kcal + g\ lipids\ 9.02\ kcal + g\ NFE \times 3.87\ kcal \quad (2)$$

Using the ***Tukey*** test, the statistical evaluation of the differences of the means was performed.

The evaluation of the sensory quality of this product was carried out in a sensory analysis laboratory of Iasi University of Life Science by the participation of a group of forty-five students in food engineering, each receiving an individual sheet. Prior to analysis, the samples were brought to a temperature of 18-21°C, according to the provisions of the professional/product standards. The analysis of shape, appearance and color is performed in natural, diffuse light. The appearance and color were examined on the outside of the products, then on the inside, visually; the consistency was analyzed on the outside and then in the products section, with the touch analyzer and by chewing. The odor analysis was performed by simple inspiration. The tasting of the samples was done carefully, without haste, with relaxation breaks of about 2 minutes between the portions of the sample; 5-10 g of product were taken for tasting. Before and after tasting each sample, the tasters rinsed the oral cavity with drinking water to eliminate the remaining taste. The evaluation of each sensory characteristic was performed by comparing with scoring scales of 0-5 points (SP 3196-83), obtaining the total average score for all the characteristics examined by the group of tasters, and by comparing it with a scale from 0 to 20 points for weighted average score obtained after tasting (Table 1). The samples were prepared in the same way for all tasters and distributed in equal quantities, in identical vessels. As a result, the arithmetic mean obtained from the score given by all tasters for each characteristic was taken.

Table 1. Classification of the products in the appropriate quality class according to standards

Total average score	Quality class/grade obtained
18.1…20	Very good
15.1…18	Good
12.1…15	Satisfactorily
7.1…12	Unsatisfactory
4.1…7	Bad
0…4	Adulterated

Examination of sensory characteristics specific to chicken hot dog sausages followed: appearance-color, consistency, taste, smell and global assessment.

The pH value of meat was measured at 24 and 48 h post-slaughter (on chilled samples, at 2-4°C), using the digital pH meter HI99163 (Hanna Instruments Ltd., UK), with a penetration probe. Calibration of the pH meter was performed at 4.0 and 7.0 pH at ambient temperature.

RESULTS AND DISCUSSIONS

The sensory analysis (Figure 1 and Figure 2.) revealed a minimum score for product E (10.63 points/"unsatisfactory product" according to quality standards), compared to product B which obtained the best score among all the analyzed assortments (17.70 points/"good product"). The average score of sensory characteristics determined by tasting highlights differences between products, but not with very high values (Figure 1). The highest average score was obtained for appearance (4.3) for product C and the lowest for consistency (3.50) for product E.

The results of chemical analysis (Figure 3) showed very high differences between products in terms of fat content (between 13.5% and 25.1%), the variability was lower for proteins (between 16.6% and 19.3%) and water content (between 55.83% and 66.94%). The salt content had the highest value of 2.83%, exceeding the maximum standard limit, only in the case of C product.

Evaluating the information presented on the product label, it was observed that only one producer has added vegetable fibers (1.42%, the product B). Even if now consumers avoid saturated fats as much as possible, by processing meat, from a technological point of view the addition of fat is practiced, for examples the fatback (the subcutaneous fat taken from under the skin of the back of domestic pigs. A hard fat, pork fatback can be valorised whole, sliced, diced, or even ground, and it's used to add moisture, fat and flavor to a wide variety of meat products: it can also be seen as small spots of fat in salami or mortadella.

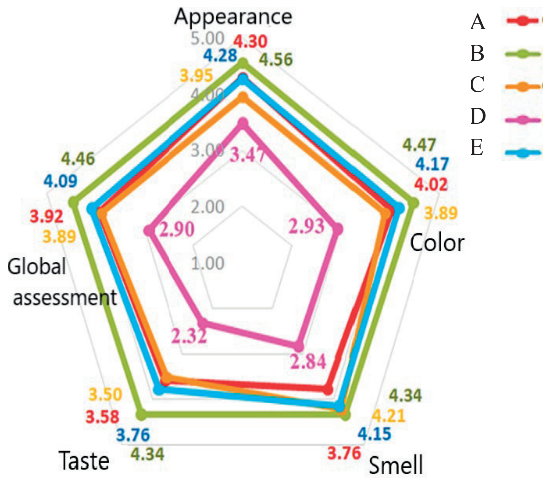

Figure 1. The results of sensory analysis

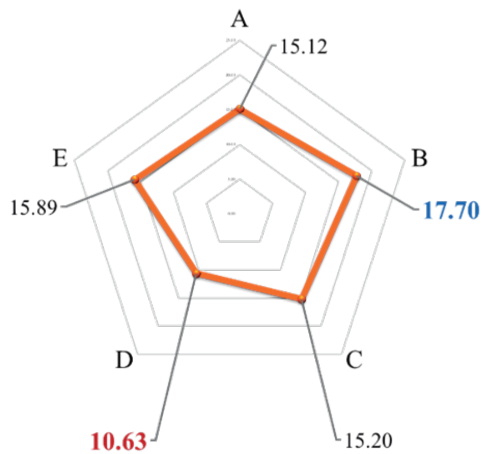

Figure 2. Weighted average score obtained after tasting

The nutritional information presented on the label by manufacturers, for 100 g of product are presented in Table 2.

Significant differences are observed at the level of protein and lipid content, respectively different energy value based on these variations for all five analyzed products.

Table 2. Nutritional information presented on the label, for 100 g of product

Nutritional information	Products				
	A	B	C	D	E
Energy value	1189 kJ/287 kcal	972 kJ/234 kcal	1031 kJ/249 kcal	1109 kJ/268 kcal	809 kJ/194 kcal
Lipids, % which	25	20	20.1	24.0	14.0
saturated fatty acids	9.1	7	7.8	8.8	3.8
Carbohydrates, %	0.5	1.42	0.5	0	1.1
of which sugars	0.2	0.36	0.5	0	0.7
Protein, %	15	13.66	16	13	16
Salt, %	1.6	2.01	1.7	2.3	2.1
Fiber, %	0	1.42	0	0	0

Following the determination of mineral substances (ash), the highest value was found for product C, 3.05%, in contrast to product A, which recorded a value of 2.08%.

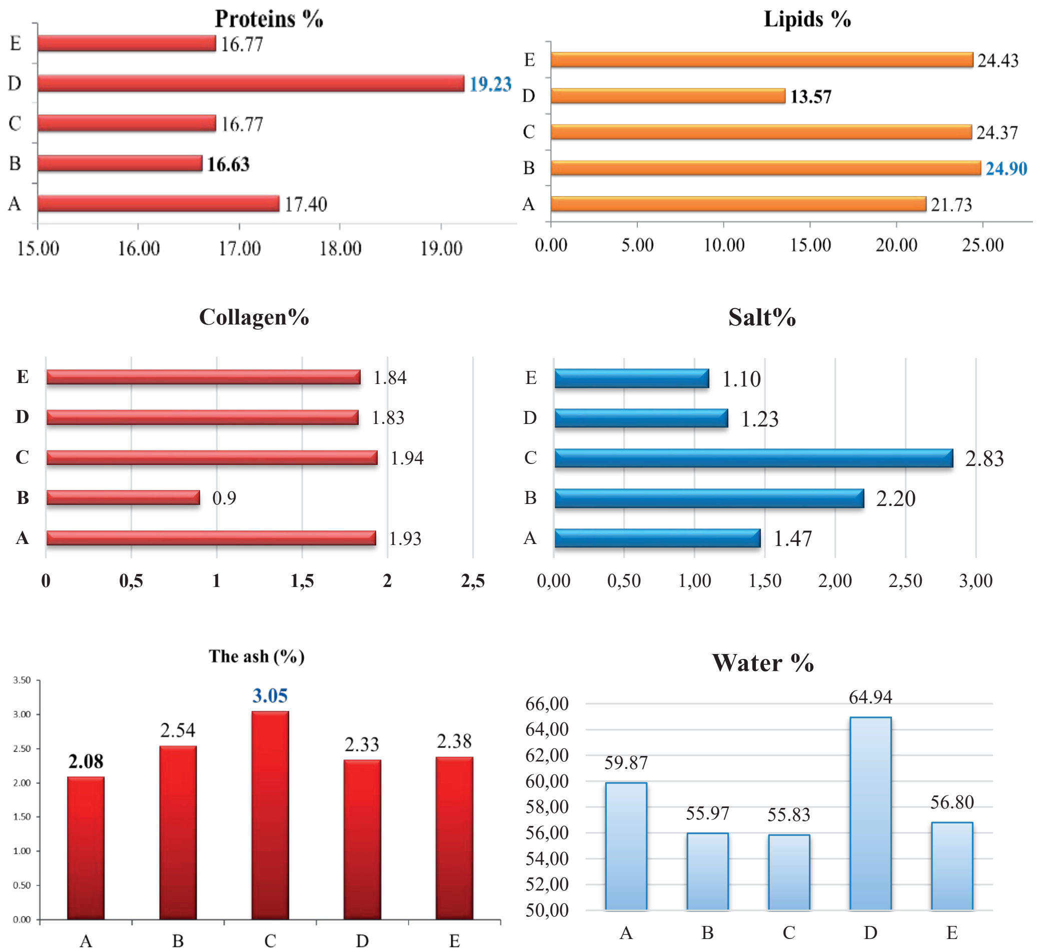

Figure 3. The results of chemical analysis of chicken hot dog sausages

According to the ingredients declared by the manufacturers, chicken breast is found in different percentages, the lowest amount being for product B (30% chicken breast), while product E has an amount of 70% chicken breast and 24% beef chickens from another anatomical region.
Although the B sausages have the least amount of meat, they were the most appreciated by the tasters following the sensory analysis. This fact may be due to the large amount of fat and salt, but also to the *monosodium glutamate* in its composition, which is a controversial flavor enhancer worldwide.
The E products, the ones with the largest amount of chicken meat, were less appreciated by the tasters, because of the poorly pronounced taste, but also the pungent, sour smell probably by the acids added in composition.
For all analyzed products, the amount of protein was supplemented with animal protein additions. The slightly higher amount of collagen for products A, B, C and D is probably due to the added chicken skin in their composition. The proportions of fatback, skin and water in the composition of sausages are not mentioned on the product label, because there are no regulations in this regard.
Comparing the results obtained with the admissibility conditions, according to standard SP 1472-85 (Figure 4), it appears that the five producers of chicken sausages respected the admissibility conditions imposed and fell within the maximum limit admitted. The exception is the C product, because the salt content had a slightly exceeded maximum limit (2.83% vs. 2.80%).

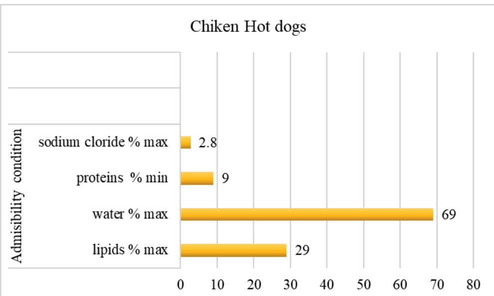

Figure 4. The admissibility conditions imposed for chicken hot dog sausages

The energy value for all studied chicken hot dog sausages (Figure 5) was close for products E, C and B, the smallest being recorded for D product. The lipids content is decisive for high or small energy value.

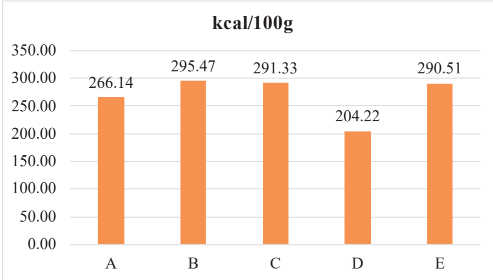

Figure 5. The energy value of studied chicken hot dog sausages

Food additives can be found in all the studied products, namely sausages A contain eleven additives, sausages B and D contain eight additives, sausages C contain seven additives, and sausages E contain four additives. Special attention should also be paid to the addition of carmine food coloring, which even if it is classified as natural pigments, can cause allergies, especially to people allergic to shellfish.
Following the study carried out, sausages are recommended to be consumed in moderation, from the point of view of the chemical composition, product E has superior properties compared to the rest of the analyzed products, but the low price and the quality class in which it was placed following the sensory analysis provide uncertainty.
Following the statistical evaluation of the differences of the means (Table 3) using the ***Tukey*** test, it was observed for product A vs. B: In lipids content: a significant difference ($p<0.05$), indicating that product A has a significantly lower lipid content than product B. This suggests a considerable difference in the fat profile between the two products.
Collagen: Significant difference ($p<0.05$), with lower values for product B, which may reflect a difference in protein structure and product quality. From the point of view of energy value, significant differences ($p<0.05$) were observed between products A and B, product A having a higher energy content. This can influence the nutritional value and energy quality of the

product. Salt, water, ash, dry matter, organic matter, NFS: No significant differences, suggesting that in these respects B and A are relatively similar D product stands out by significant differences from most other groups, having higher values for multiple characteristics. A and E showed significant differences in some measurements compared to other groups, while B and C are relatively similar to each other with limited significant differences. These results suggest that D and A are the most distinct in terms of nutritional profile and physical characteristics, and B and C are closer to each other.

A vs. C: Ash: Significant difference ($p<0.05$), indicating that C has a significantly higher ash content than A, which may reflect a difference in mineral content and manufacturing process.

Other measurements (lipids, proteins, collagen, salt, water, dry matter, organic matter, NFS, crude energy): No significant differences. This suggests that most of the chemical and nutritional characteristics are comparable between A and C.

A vs. E: Lipids, collagen, ash, NFS: Significant differences ($p<0.05$), with E having lower values in all these characteristics compared to A. This indicates a difference in the lipids content, protein quality and energy values of the products. Salt, water, dry matter, organic matter, gross energy: No significant differences, suggesting that these characteristics are relatively similar between the two groups.

A vs. D: Lipid, protein, collagen, salt, water, dry matter, organic matter, gross energy: All these characteristics show significant differences ($p<0.05$), with D having higher values compared to A. This suggests that D is significantly different in terms of nutrient and energy content, having a higher value in most aspects.

B vs. C: Collagen: Significant difference ($p<0.05$), C having higher collagen content compared to B, which may reflect a difference in protein quality. Other measurements (lipid, protein, salt, water, ash, dry matter, organic matter, NFS, crude energy): No significant differences, suggesting similarities between B and C in these aspects.

B vs. E: Salt, ash: Significant differences ($p<0.05$), having higher values in salt and ash compared to B. This could indicate differences in the mineral composition and salt content of the products. Other measurements (lipid, protein, collagen, water, dry matter, organic matter, gross energy): No significant differences, suggesting similarities in most other characteristics.

B vs. D: Lipids: Significant difference ($p<0.05$), D having a significantly higher lipid content compared to B. Other measurements (protein, collagen, salt, water, ash, dry matter, organic matter, gross energy): No significant differences, indicating that, apart from lipids, the other characteristics are similar between B and D.

C vs. E: Collagen, lipids: Significant differences ($p<0.05$), C having higher values for collagen and lipids compared to E. This suggests differences in protein and fat content between the two products. Other measurements (salt, water, ash, dry matter, organic matter, gross energy): No significant differences, suggesting similarities in these aspects.

C vs. D: Lipids, proteins, collagen, salt, water, dry matter: All these characteristics show significant differences ($p<0.05$), with D having higher values compared to C. This underlines significant differences in composition and nutritional value between the two products.

E vs. D: Lipid, protein, salt, water, dry matter, organic matter, NFS, crude energy: Significant differences ($p<0.05$), D having higher values in all these characteristics compared to E. This suggests that D is consistently richer in nutrients and energy than E.

The high acidity (Figure 6) in chicken sausages A may be due to the fact that ascorbic acid and lactic acid were used in their composition as an antioxidant and preservative.

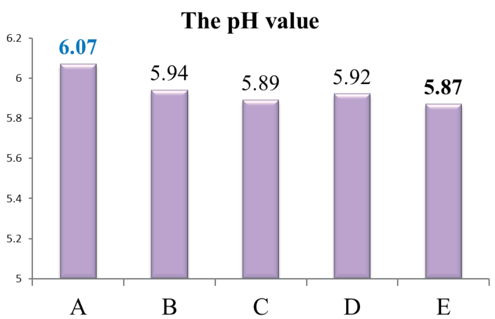

Figure 6. The average value of pH

Table 3. The statistical significance of differences regarding the chemical composition of some assortments of chicken hot dog sausages (Tukey test)

	Lipids		Proteins		Collagen		Salt		Water		Ash		Dry matter		Organic matter		NFS		Energy value	
	p value	Signif.	p value	Signif.	p value	Signif.	p value	Signif.	p value	Signif.	p value	Signif.	p value	Signif.	p value	Signif.	p value	Signif.	p value	Signif.
A - B	7.967	*	1.900	ns	1.930	*	0.833	ns	6.267	ns	0.574	ns	6.267	ns	6.841	ns	0.774	ns	66.742	ns
A - C	4.800	ns	1.133	ns	0.900	ns	0.100	ns	3.767	ns	1.030	*	3.767	ns	4.797	ns	1.130	ns	42.831	ns
A - D	5.333	*	1.267	ns	1.940	*	0.533	ns	4.233	ns	1.536	*	4.233	ns	5.769	ns	1.703	*	49.287	ns
A - E	16.133	*	3.733	*	1.830	*	1.067	*	12.800	*	0.822	ns	12.800	*	13.622	*	1.222	ns	134.309	*
B - C	3.167	ns	0.767	ns	1.030	*	0.733	ns	2.500	ns	0.456	ns	2.500	ns	2.044	ns	0.356	ns	23.911	ns
B - D	2.633	ns	0.633	ns	0.010	ns	1.367	*	2.033	ns	0.962	*	2.033	ns	0.928	ns	0.929	ns	17.455	ns
B - E	8.167	*	1.833	ns	0.100	ns	0.233	ns	6.533	ns	0.248	ns	6.533	ns	6.781	ns	0.448	ns	67.567	ns
C - D	0.533	ns	0.133	ns	1.040	*	0.633	ns	0.467	ns	0.506	ns	0.467	ns	2.972	ns	0.573	ns	41.366	ns
C - E	11.333	*	2.600	*	0.930	*	1.000	*	9.033	*	0.229	ns	9.033	*	4.737	ns	0.682	ns	43.656	ns
D - E	10.800	*	2.467	*	0.110	ns	1.633	*	8.567	*	0.734	ns	8.567	*	7.709	*	1.255	*	85.022	*

Statistically significance (Signif): * = significant differences; ns = insignificant differences.

Table 4. The declared ingredients and additives of some assortments of chicken hot dog sausages

Ingredients	A	B	C	D	E
Chicken breast	**50%**	**30%**	**55%**	**70%**	**40%**
Pork fatback	**?**	**?**	**?**	**-**	**?**
Chicken skin	**-**	**?**	**?**	**-**	**-**
Other ingredients	pork (30%), animal protein from pork, sugars	milk protein, modified potato starch, ***vegetable fibers***, sugars	milk protein, animal protein from pork, vegetable fibers, sugars	chicken (24%), milk protein (0.3%)	milk protein
Water	?	?	?	?	?
Salt	1.6	2.01	1.7	2.10	2,3
Spices	spice and flavoring extracts	natural spices	spice extracts, natural flavors	pepper, spice extracts, flavors	?
Additives	diphosphates, triphosphates, ascorbic acid, sodium ascorbate, sodium erythorbate, sodium lactate, E250 sodium nitrite, E262 sodium acetate, E270 lactic acid, E327 calcium lactate, smoke flavors	Na and K di-polyphosphates, E621 monosodium glutamate, ascorbic acid, E316 sodium isoascorbate, sodium nitrite, smoke flavors	diphosphates, sucrose, ascorbic acid, vegetable extract of red pepper, E120 carmine, sodium nitrite	sodium ascorbate, citrus fibers, sodium nitrite, smoke flavor	diphosphates, sodium acetate, sodium nitrite, E621 monosodium glutamate, ascorbic acid, sodium ascorbate, carmine, smoke flavors

The difference between the prices can be explained by the quality of the packaging, by the quality of the raw and auxiliary materials used, but also by the popularity of the brand at national level (Figure 7).

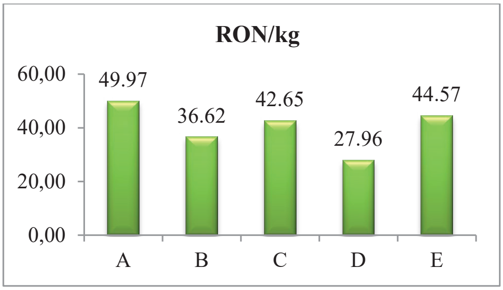

Figure 7. The average price of the products/kg from 2023

According to the most recent studies in the field of human nutrition, unfortunately the presentation of products with addressability, especially to children, is detrimental to their health if this type of product is consumed very frequently and in large quantities, without having a diversified diet (rich in fresh fruits and vegetables, whole grains, legumes and/or other sources of vegetable fiber).

The competent authorities should introduce updated regulations aimed at protecting the health of consumers in terms of saturated fat content and added additives, as it is well known the fact that the meat sausages /ultra-processed meat are declared potentially carcinogenic by the World Health Organization.

In recent years, however, there has been an improvement in the quality of the products available on the Romanian market, with the concept of a "*clean label*" appearing on the label of different food products, which also applies to the some assortments of chicken hot dog sausages.

CONCLUSIONS

The results of the sensory analysis revealed a minimum score for product D (10.63 points/ "unsatisfactory" product according to quality standards), compared to product B which obtained the best score among all the analyzed assortments (17.70 points/"product good"). Sausages from manufacturer B (with the least amount of meat) were the most appreciated by the evaluators following the sensory analysis, probably based on the high amount of fat and the addition of monosodium glutamate in its composition (an extreme controversial flavor enhancer worldwide). The results of the chemical analyses revealed high differences between producers at the level of fat content, 11.6 percentage points (13.5% vs. 25.1%), for the protein content the variability was less, 2.7 percentage points (16.6% vs. 19.3%), but also for the amount of water, 9.3 percentage points (57.2% vs. 66.5%). The salt content had a slightly exceeded maximum limit for product C (2.83% vs. 2.80% according to the standard), and the other products fell within the maximum allowed limit. For lipids, proteins and moisture, the products fell within the limits of the standard. The highest pH value was recorded for product A (pH = 6.07), probably due to the lactic and ascorbic acids in the composition. Of all the manufacturers analyzed, product D recorded the lowest energy value, due to the low fat content in the composition.

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WATER ACTIVITY AS A STRUCTURING PARAMETER OF THE HYSTERESIS CYCLE AND ITS ROLE IN FOOD STABILITY

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Abstract

In food science, water sorption isotherms are often used to understand the stability of food preserved in different conditions. Water activity is defined as the ratio of the vapor pressure of water in a material (p) to the vapor pressure of pure water (po) at the same temperature. In food, water activity (a_w) explains how water affects the rate of microbial growth and many other chemical reactions. The paper aimed to study aw as an indicator of structuring the hysteresis cycle, its role in the changes that occur in several dried fruits in the Albanian market and to reach the hysteresis cycle through the determination of aw values in adequate conditions. Referring to physico-chemical indicators as protein, fat, and acidity, they were found to be within the limits allowed by law. So, there was no visible impact on the change of these indicators during the storage period. From the results obtained for walnuts as a specific case, it was observed that due to the dependence of moisture content on aw, the adsorption/desorption isotherms do not overlap, which refers precisely to the hysteresis cycle. It was possible to obtain a second-order desorption isotherm, with a very good approximation.

Key words*: food safety, hysteresis cycle, moisture, water activity, Water Activity (aw) Meters.*

INTRODUCTION

Food preservation involves the action taken to maintain the nature of a selected food and/or its desired properties, with the aim of keeping it fresh and safe to the consumer within a certain period of time. A stable food product can be developed by applying different processing techniques and by keeping it in appropriate conditions. Food stability determination from a scientific basis rather than empiricism is a challenge to food scientists and engineers (Sandulachi et al., 2012).

Water is a very important component in foods. For a long time, the industry has known how important it is to check the free water. Water activity (a_w) measurement forms the basis of this and provides important information about the safety and quality of a product. Finally, it provides information regarding the possibility of microbiological growth on the surface. Studies for the evaluation of water activity in food products and their conclusions, are part of the scientific research on the preservation, stability and shelf life of food products. Water activity (a_w), combined with temperature and pH have a significant effect in microbial growth in food (Peleg et al., 2021).

In order for a food to have an appropriate shelf life without relying on refrigerated storage, it is necessary to control either its acidity level (pH), the level of water activity (a_w) or a suitable combination of the two. This can effectively increase the product's stability and make it possible to predict its shelf life under known environmental storage conditions. (Sandulachi et al., 2012).

The water activity (a_w) of a food is the ratio between the vapor pressure of the food itself, when in a completely undisturbed balance with the surrounding air media, and the vapor pressure of distilled water under identical conditions $a_w = (p/p_o)$ T. Aw also defined as the ratio of the "the sum of the partial pressures that the water vapors coming out of the components of a food product, exert on the product itself and the vapor pressure of distilled water under identical conditions". The term "water activity" (aw) was developed to reflect the intensity with which water associates with various nonaqueous constituents. Relative vapor pressure-RVP is related to the percentage

of relative humidity in equilibrium conditions (% ERH) of the product environment as follows: RVP = (p/po) T = % ERH/100. Relative vapor pressure (RVP) is the name for (p/p_0) T (Damodaran et al., 2007).
The recognition of a_w as a storage and stability factor for dried fruit-nuts, was an incentive for the experimental work presented in this publication.
Nuts have water activities that are generally less than 0.7. This group are usually described as dry fruits with an edible seed and a hard shell, with cashews (*Anacardium occidentale*), walnuts (*Juglans regia*), almonds (*Prunus dulcis*), chestnuts (*Castanea sativa*), pistachios (*Pistacia vera*), and hazelnuts (*Corylus avellana*) as the ones with higher production worldwide. It is known that nuts are a good source of many nutrients, including monounsaturated and polyunsaturated fatty acid profiles, vitamins E and K, selected minerals such as magnesium, copper, potassium, and selenium, dietary fibers, carotenoids, and phytosterols with potential antioxidant properties (De Souza et al., 2017). Nuts are a rich source of proteins and essential amino acids (Gonçalves et al., 2023). It is important to mention the beneficial effects of walnuts due to the fatty acid profile found in walnut oil, in particular the presence of ω-3 and ω-6 PUFA that are essential dietary fatty acids. These are the nonaqueous constituents that the value of a_w depends on (Boaghi et al., 2019). Our study was focused on the experimental determination of the physicochemical characteristics of dried fruits. Hysteresis can have a significant effect on the chemical reactions in food (Zomorodian et al., 2007). In this research we have considered the study of the hysteresis cycle specifically for walnuts.
A plot of water content (expressed as a mass of water per unit mass of dry material) of a food vs. (p/p_0) T is known as a Moisture Sorption Isotherm (MSI) (Damodaran et al., 2007). However, not all of the products resulted in the same curve type. The classification of the curve type could explain the properties of that product. Moisture sorption isotherms exhibit a variety of shapes, many of which are amenable to at least qualitative interpretation (Andrade et al., 2011). Sorption isotherms classified according to their shape and processes, establish five different types (Blahovec & Stavros, 2009).
In food science, isotherms are primarily used to understand the effects of drying, processing and storage on the quality of food products (Nollet et al., 2008; Bell & Labuza, 2000; Hay et al., 2022). In particular, by fitting a theoretical model such as the Brunauer-Emmett-Teller (BET) (Staudt et al., 2013) or Gugenheim-Anderson-de Boer (GAB), they are often used to determine what is known as "the water in monomolecular layer" (M_m). In theory, as moisture content increases above M_m, more and more water become available to act as a solvent and thereby facilitate the change reactions of food products. According to theoretical data (Damodaran et al., 2007), the experimental curves follow the same path in sorption and desorption, regardless of the deviations that depend on the type of food product or raw materials and the processes to which they are subjected.
Water activity, unlike water content, can determine a food's shelf stability. The microorganisms are potential sources of contamination and spoilage in different values of water activity (Majumdar et al., 2018; Macri et al., 2020). Hence several preservation processes are used to extend the stability of foods by reducing the moisture content to levels below those required by micro-organisms for survival and reproduction (Liu et al., 2022; Racchi et al., 2020). Even at low pH values and low a_w, certain yeasts and molds species might pose a risk to the stability of Intermediate Moisture Foods (IMF) (Vermeulen et al., 2015). There are different approaches to conservation and stability of fresh fruit products or dried fruits. On the other hand, the stability of shelf-stable products (SSP) is achieved with a combination of factors, (including a_w) and with good production and distribution practices, (including the selection of packaging).

MATERIALS AND METHODS

The experimental work was structured in the selection and preparation of the samples, sensorial and physicochemical characterization, and finally the construction of the sorption/desorption isotherms of linearized hysteresis cycle. Several types of dried fruits, with a wide use in Albanian market were selected. The samples of almonds, hazelnuts, pistachios, walnuts, salted peanuts vacuum packaging, old walnuts and unsalted peanuts were evaluated. First, a sensorial assessment of all samples was carried out. Products were considered safe, at first sight, in terms of texture, color, aroma, and taste. Each sample was cut first, thus preparing for physico-chemical evaluation and determination of water activity. Physicochemical characterization included determination of moisture, fat using the Soxhlet extraction method, determination of nitrogenous substances and proteins using improved Kjeldahl method (Sáez-Plaza et al., 2013) and titratable acidity (expressed in oleic acid) of grinded samples based in the formula below (Nielsen et al., 2010):

$$\%\,acid\left(\frac{wt}{wt}\right) = \frac{N\ x\ V\ x\ Eq\ wt}{W\ x\ 1000}x\ 100$$

Where:
N = normality of titrant, usually NaOH (mEq/ml)
V = volume of titrant (ml)
Eq. wt. = equivalent weight of predominant acid (mg/mEq)
W = mass of sample (g)
1000 = factor relating mg to grams (mg/g) (1/10 = 100/1000).

The determination of water activity was performed using the NOVASINA AG, CH-8853-Lachen. Labstart-aw device.

The determined water activity values were accompanied by the determination of moisture under the same conditions for each sample. Moisture was determined by the classic method, with drying in a thermostat until constant weight, at a temperature of 105°C. In order to study the hysteresis cycle and to evaluate the stability of the sample, a walnut sample was used by building a work package with sorption and desorption of water from the sample cut in small pieces, for a period of time of 1, 3 and 6 hours. The sample was previously dried and the moisture and water activity were measured periodically. Then it was moistened again with 25 ml water and the periodic increase in humidity and the respective water activity were measured in order to observe the trend of water sorption isotherms.

RESULTS AND DISCUSSIONS

The values of average moisture for all samples are presented in the Figure 1 and Table 1.

It is observed that walnuts have the highest percentage of the moisture. Old walnuts have a moisture reduction of 12.63% compared to fresh walnuts. This decrease is understandable referring to the long storage time. The old walnuts have been stored for 2 years in a dried place and this reduction has influenced the inhibition of the visible development of microbial loads. Salted peanuts in vacuum resulted in an increase in the moisture by 50% of the value, while it was expected that the salt would play the role of protector by changing the osmotic pressure. Apparently, opening the package, stimulates immediate absorption of water. Another factor could be the use of very dry storage places for unsalted peanuts. This situation should be evaluated with a future experimental work, intended only for this type of product, its storage and packaging forms.

Data obtained from the analysis of the titratable acidity, fat and protein content in dried fruits are presented in Table 2 and Figure 2.

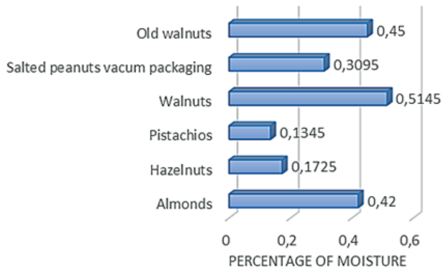

Figure 1. Moisture values in % for different types of dried fruits

Table 1. Moisture values in % for different types of dried fruits

No.	Samples	Moisture, %
1	Almonds	4.652
2	Hazelnuts	2.697
3	Pistachios	1.7105
4	Walnuts	5.232
5	Salted peanuts vacum packaging	2.6825
6	Old walnuts	4.2065

The acidity values presented in Table 2 and it is observed a very low acidity for all samples, so in reality acidity does not represent an important indicator for their physicochemical changes. The selected samples are neutral to slightly alkaline. This also explains the fact that many of them, for example pistachios are used as satisfactory choices for the people with acid reflux, or with problems in the digestive tract (Mandalari et al., 2022). The samples show also high percentages of fat. They are considered as fatty woody fruits. The highest percentage of fat it is observed in walnuts and this an expected result referring to the theoretical values of triglycerides in dry woody fruits (average value 65.21%). A result under discussion is that of the fat in a local product hazelnut, which after verification and repetition, comes out again with this value. Even the protein values for all samples are within the expected range.

Table 2. The determination of acidity, fat and proteins in the selected samples

No.	Samples	Acidity, %	Fat, %	Proteins, %
1	Almonds	0.2794	47	21.86
2	Hazelnuts	0.1397	28.9	21.97
3	Pistachios	0.4224	50.41	22.36
4	Walnuts	0.2808	64.76	20.04
5	Salted peanuts vacum packaging	0.0823	56.28	20.63
6	Old walnuts	0.2220	59.62	17.20

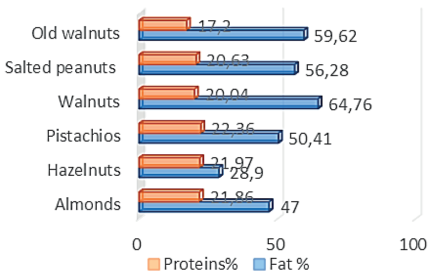

Figure 2. Fat and proteins in the selected samples

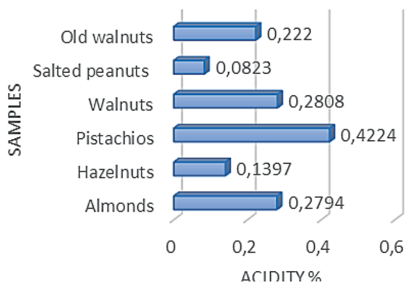

Figure 3. The percentages of acidity of the selected samples

Water activity was determined using the NOVASINA AG. Device.

For each measurement, the temperature was controlled. The experiments were performed in an area with a controlled temperature of 22-25°C. Water activity was measured three times for the same sample and the average values are presented in Table 3 and the Figure 4.

Table 3. Values for water activity

Nr.	Samples	Aw
1	Almonds	0.58
2	Hazelnuts	0.52
3	Pistachios	0.47
4	Walnuts	0.70
5	Salted peanuts	0.50
6	Old walnuts	0.57

Almonds, hazelnuts, pistachios, salted peanuts and old walnuts have the values of aw at the levels of the lower limit of mould development (a_w = 0.5-0.6). These are expected values and explain the contamination of these products by these microorganisms, especially *Aspergillus* sp. as an aflatoxin-producer in long time preserved woody fruits, in high humidity conditions.

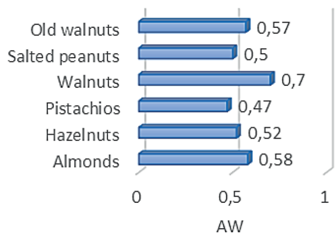

Figure 4. Water activity values for the selected samples (results of Table 3)

Walnuts have a higher value of water activity and unsalted peanuts have a very low value, slightly higher than that of the monomolecular layer. There are no values that correspond to the development of yeasts or bacteria (respect-tively a_w >0.8 and a_w >0.9). In fact, the low values of aw in this group, make them resistant to the presence of endemic microorganisms, while the presence of some identified pathogens, such as *Salmonella*, *Clostridium, Listeria*, etc. (Brar et al., 2018), is related to the lack of hygiene in collection, processing, distribution as well as consumption.

The construction of the specific hysteresis cycle based on aw-moisture relationship was considered for walnut samples (Table 4).

Table 4. A_w-moisture dependence for walnuts in the conditions of adsorption and desorption of the aqueous phase

	Aw	Moisture (humidity) %	
Absorption values	0,54	2,93	
	0,96	16,99	
	0,95	17,8	
	0,96	19,65	
	0,95	22,006	
	0,95	8,88	Desorption values
	0,89	6,147	
	0,83	4,105	
	0,51	2,76	

The cycle provided an adsorption process that approximately responded to a first-degree equation, while the desorption, a real deviation and responded to a typical second-degree equation $y = kx^2+bx+c$. In the case of adsorption, the graphical approximation is clear with the line of linearity.

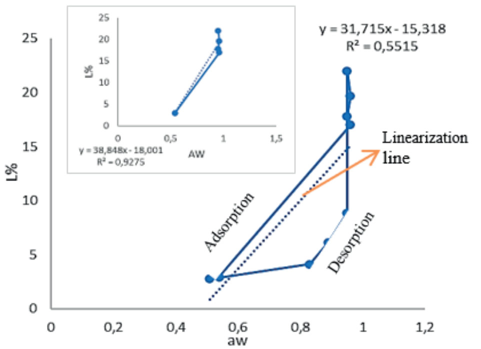

Figure 5. The adsorption/ desorption cycle obtained for walnuts (Inner graph - the adsorption isotherm only)

Walnuts are often consumed in a period of time, away from their collection and processing. So, in the risk of microbiological and physicochemical pollution, desorption is of particular interest, since their storage in an open environment can be done under different conditions of working practices. For this reason, desorption process was used for a test sample, and it was studied without changing the water absorption conditions, to verify the shape of the desorption lines. The results are presented in the Table 5 and Figure 6. Differences in the values of water activity are related with the tissue structure and porosity of the selected sample.

Table 5. Aw-moisture dependence of walnuts in the conditions of the desorption study

	Aw	L%	
The adsorption of the existing sample	0,54	2,93	
	0,96	16,99	
	0,95	17,8	
	0,96	19,65	
	0,95	22,006	
	0,95	18,97	Desorption of a new sample
	0,95	12,41	
	0,83	4,8	
	0,56	3,1	

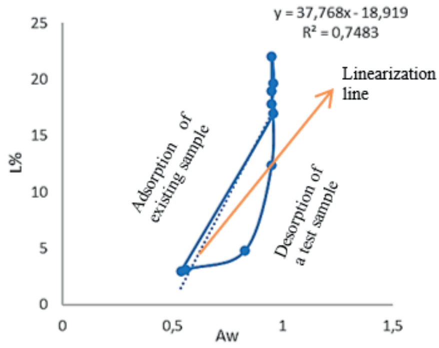

Figure 6. The cycle of adsorption-desorption for walnuts

Comparison of the Figures 5 and 6 clearly represents and verifies the same shape of desorption line, so this cycle can be used in any case for walnut samples, also for the linearization of the desorption curve, using B.ET. Equation (Damodaran et al., 2007) as below:

$$\frac{a_w}{m\,(1-a_w)} = \frac{1}{m_1 c} + \frac{c-1}{m_1 c}\,a_w$$

Where:
m - moisture, g H_2O/g dry matter
a_w - water activity
m_1 - BET monolayer value
c - a constant.

CONCLUSIONS

The evaluation of water activity is a very convenient method to observe the condition of dried fruits in water sorption and desorption processes, so their drying processes can be evaluated both by determining the moisture and the a_w values. Theoretical and experimental study of water activity concept, allows evaluating the choice of method of preserving food.

The evaluation of the physicochemical parameters of dried fruits in conservation, it was found to be within the limits allowed by law. This shows that for the samples in this study, there was no visible impact on the change of these parameters during the storage period. However, water activity, pH, temperature, and other parameters, have a direct impact on the microbial growth, thus a_w and pH are two of the most important parameters taken into consideration.

The high content of proteins, fats and dry matter brings the respective value of a_w below 0.6-0.7. During the study of the hysteresis cycle for walnuts, it was possible to obtain a second-order desorption curve and a straight line of the type $y = kx+b$ for the adsorption process, with the best possible approximation ($R^2 = 0.9275$).

From the results presented also graphically, an experimental study extended in time for walnuts in storage is suggested. A comparison of a_w values is proposed to be evaluated, in order to observe if there have been created conditions to develop harmful microorganisms, mainly aflatoxin-producing moulds taking into consideration also pH as a very important indicator. It is also suggested that the evaluation of a_w can be accompanied by respective microbiological tests, in order to analyse the microbial growth in the same samples under study and to isolate and identify the specific strains of interest in this field.

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EFFECT OF SEX ON CHEMICAL COMPOSITION AND MEAT QUALITY OF JAPANESE QUAIL (*Coturnix japonica*)

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Abstract

Japanese quail (Coturnix japonica) is used for egg production, as laboratory animals, in amateur breeding as an ornamental bird and for meat production. Quail meat is becoming more and more popular in Poland. The aim of the study was to analyze the chemical composition of Japanese quail meat from a Polish breeder, taking into account the sex of the birds. The research was carried out on 20 chilled quail carcasses - 10 females and 10 males. Meat pH, basic chemical composition, amino acid levels, fatty acid profile, cooking loss and color parameters were determined in the breast muscle. The analyzed meat was characterized by high cooking loss. The chemical composition of quail meat, especially the high protein content and low fat content, make this meat characterized by a low caloric value. The meat of males was characterized by a higher content of lysine and glutamic acid and a lower content of histidine, arginine, tyrosine and methionine compared to the meat of females. The gender of quails had no significant impact on meat quality parameters.

Key words: *chemical composition, color, Japanese quail, meat, quality.*

INTRODUCTION

The common quail (*Coturnix coturnix*) is a representative of the genus Quail (*Coturnix*) belonging to the family Curlews (Phasianidae) and order Burrowing Owls (Galliformes), which does not lead a sedentary lifestyle and spends the winter in the Sahel (Kosicki et al., 2014). In Poland, it is under strict species protection. It is on the Red List of Polish Birds, where it has been classified as a vulnerable species (VU), and agricultural intensification is considered to be the cause of its endangerment. The Japanese quail (*Coturnix japonica*) belongs to the same genus as the European field quail (*C. coturnix*). It was domesticated in the sixteenth century in China, but it was not until the twentieth century that breeding work began in Japan to improve the utility value of these birds. In Poland, interest was taken in the breeding of Japanese quail in 1963 after the first flocks were imported by Professor Jerzy Szuman. Since the 1990s, a steady increase in interest in this species has been observed (Kraszewska-Domańska, 1978). Japanese quails are used in several ways. Meat use is of interest to breeders in China, Europe and the USA, while quail egg production takes place mainly in China, Japan and Brazil (Carvalho et al., 2020; Ionita et al., 2011; Minvielle, 2004). In France and the USA, Japanese quail are used for hunting purposes (Minvielle, 2004). Established colour varieties are of importance for amateur breeding, also from an exhibition point of view, especially in the USA and Western Europe. Japanese quails are also used as a model organism and laboratory animal in many research centres (Quaresma et al., 2022). The main producers of quail meat are China, the USA and Europe (Tserven-Gousi & Yannakopoulosa, 1986). Quail meat is tasty, tender and healthy, as well as being lean and low in calories. The quality and composition of this meat depends, among other factors, on the variety, slaughter age, and diet (Genczew, 2003; Jakubowska & Karamucki, 2020; Vargas-Sánchez et al., 2019; Sabow, 2020). Pharaoh quails, selected in the USA, are characterised by their wild colouration and highest body weight of all varieties. The breed is suitable for broiler production and is characterised by a very well-developed pectoral muscle. Females are larger and heavier than males (Kraszewska-Domańska, 1978).

The aim of this study was to analyse the effect of the sex of Pharaoh quails, on the chemical composition and quality of meat.

MATERIALS AND METHODS

The study was conducted on 20 chilled quail carcasses (10 females and 10 males) of the Pharaoh breed, slaughtered at 10 weeks of age. The birds were fed Starter feed (protein 25.5%, lysine 16.7 g, methionine 7 g, threonine 9 g, 12.5 MJ of metabolizable energy) followed by Grower feed (protein 22%, lysine 14.4 g, methionine 6 g, threonine 7.6 g, 12.15 MJ of metabolizable energy). Quails were slaughtered in an official slaughterhouse, and stored under refrigeration (<5°C) during 24 h preceding the delivery at the laboratory. Carcasses were weighed, and afterward, breast muscles (*M. pectoralis major* and *M. pectoralis minor*) from skinned quail carcasses, of all groups included in the study, were collected from both carcass sides. Thermal losses during roasting were determined on the right breast muscle. Thermal treatment in an electric furnace was carried out at a temperature of 180 ± 2°C until reaching a muscle temperature of 72 ± 2°C. The temperature inside the muscles was measured with a digital thermometer using a probe needle. After heat treatment and cooling on ice, cooking loss was determined from meat weight loss. The left breast muscle was subjected to physical and chemical analysis. The following analyzes were performed in raw meat: (all analyzes were performed in duplicate):

- Water content according to the standard PN-ISO 1442:2000 (Polish Committee for Standardization, 2013);
- Fat content according to the standard PN-ISO 1444:2000 (Polish Committee for Standardization, 2013) (Tecator's Soxtek HTZ-2 apparatus);
- Protein content by Kjeldahl method PN-75/A-04018 (Polish Committee for Standardization, 2002) (Büchi Labortechnik AG, a B426 mineralization furnace and a B339 distiller made in Switzerland);
- Total ash content according to the standard PN-ISO 936:2000 (Polish Committee for Standardization, 2013);
- Total carbohydrates content was calculated assuming that the all total solids and water stand for 100%.

The energy value was calculated using conversion factors, according to the Guide to Regulation (EC) No. 1169/2011.

Meat colour was determined using a Konica Minolta CM-600d spectrophotometer (Minolta Co., Ltd., Tokyo, Japan) with a 50-mm diameter measuring head in the CIE L*a*b* system, where the L* parameter corresponds to the degree of lightness (L* = 0: black, L* = 100 : white), a* and b* are colour components (a*>0 red, a*<0 green, b*>0 yellow, b*<0 blue). The chromametre was calibrated against a white tile (Y = 93.8, x = 0.3136, y = 0.3192) (CIE, 1986).

Fatty acid profile was determined by two analytical methods: lipid extraction from meat according to Folch et al., (1957), and esterification according to AOAC (1995). The fatty acid methyl esters were separated by gas chromatography using a Trace GC Ultra (Thermo Electron Corporation, Milano, Italy) with a flame ionization detector (FID) using Supelcowax 10 column (30 m × 0.25 mm × 0.25 μm). The separation conditions were as follows: helium as the carrier gas, 1 mL/min; FID detector temp. 250ºC; injector temp. 220ºC; oven temp. Was held at 160ºC and increased (3ºC/min) to 210ºC (35 min); split ratio 10 mL/min. To the obtained fat (around 10 mg), 0.5 mL of 0.5M KOH in methanol was added and heated at 85ºC, after which 1 mL of 12% BF3 in methanol was added and reheated at 85ºC. After cooling to room temperature, 1 mL of hexane and 5 mL of saturated NaCl solution were added. 1 μL of the solution was injected on the chromatograph.

Individual fatty acid methyl esters (FAME) were identified by comparing with a standard mixture of 37 FAME components (Supelco Bellafonte PA, USA, Sigma-Aldrich Co. St. Louis, MO, USA) and CLA isomers (Sigma-Aldrich Co. St. Louis, MO, USA).

Determination of the amino acid profile

Determination of the amino acid profile was carried out by reversed-phase liquid chromatography using the ACCQ Tag analytical kit from Waters (Millford, MA, USA). Hydrolysis of approximately 30 mg of the sample was carried out with 4 mL of 6M HCl (POCH, Poland) and the addition of 15 μL of phenol (Sigma Aldrich St. Louis, MO, USA) at 110°C for 24 hours. The sample was sealed under a nitrogen atmosphere. The resulting hydrolysate was filtered through 0.45 μm syringe filters and then dried using nitrogen. The sample thus prepared, after appropriate dilution, was subjected to an derivatization procedure according to Waters' recommendations. For this purpose, 10 μL of the sample was mixed with 70 μL of borate buffer (pH in the range 8.2 to 9.0) and then 20 μL of 6-aminoquinolyl-N-hydroxysuccinimidylcarbamate (AQC) reagent at a concentration of 3 mg/mL acetonitrile was added. Standards (company Waters USA) were handled analogously. Chromatographic separation was performed using a liquid chromatograph from Thermo Scientyfic: a Dionex Ultimate 3000 equipped with an LPG - 3400 SD gradient 4-channel pump, a WPS 3000 TSL autosampler and a FLD-3400RS 4-channel fluorescence detector. The column used for the analysis was a Nova -Pak C 18, 4 μm (150x 3.9 mm) column from Waters. Separation temperature 37°C. Elution was carried out in a two-component gradient and a flow rate of 1 mL/min: eluent A acetate-phosphate buffer pH = 5.2, B acetonitrile/water 60:40. Gradient: 0 min - 100% A, 0.5 min - 98% A, 15 min - 93% A, 19 min - 90% A, 32 min - 67% A, 33 min - 67% A, 34 min - 0% A, 37 min -0% A, 38 min - 100% A, 64 min - 100% A, 65 min - 0% A. Detection Excitation wavelength 250, Emission wavelength 395. Quantitative analysis was performed using 1-point calibration (using an analytical standard of 100 pmol each). Development of results using Chromeleon 7.0 software. All reagents from Waters (Millford, MA, USA) kit: Standards, borate buffer, AQC. Eluent acetate-phosphate buffer (pH 5.2). Water, Acetonitrile Sigma Aldrich (St. Louis, MO, USA)

Statistical analysis

The results were analyzed with ANOVA and present as means with standard deviation. The calculations were performed with licensed software - Statistica version 13.1. (2019). The least square means and the standard deviation (SD) are presented in tables. Significance was declared at *P*< 0.05.

RESULTS AND DISCUSSIONS

Pharaoh quails are among the meat breeds suitable for broiler production. The carcass weight of 10-week-old birds ranged from 163 g in males to 179.8 g in females (Table 1). The content of hydrogen ions in the breast muscles after 24 hours of cooling (pH_{24}) was within the limits 5.70-5.71 and was similar to the results obtained by Genchev et al. (2005), who analyzed the pectoral muscles of 31-day old Pharaoh quails (5.61-5.66). These results are consistent with other publications in which the pH range of quail meat ranges from 5.30 to 6.58 (Genchev et al., 2008; Zerehdaran et al., 2012; Narinc et al., 2013). The breast muscles of males were characterized by a non-significantly higher value of color parameters a* and b*. According to Wilkanowska & Kokoszyński (2011), the L* value (color lightness) of breast muscles of Pharaoh quails was higher in birds slaughtered at 33 days of age (57.0). In quails reared until the 56th day of life, Boni et al. (2010) found lighter muscles (L*-61.54), greater yellowness (b*-19.81), less redness (a*-6.84) compared to the birds analyzed in our study. Cooking loss of the breast muscles of female pharaoh quails was greater (35.2%) than that of males (34.11%). These results are similar to those obtained by Tarasewicz et al. (2007) (35.6-35.8%) and Gardzielewska et al. (2012) (35.6-37.37%), but too high compared to the study of Kaye (2014), who found thermal leakage of 17.7-20.3%, the study of Nasr et al. (2017) (19.21-20.6%) and the study of Genchev et al. (2008) - 21.68% for quail pectoral muscle.

Table 1. Slaughter and quality characteristics of meat from breast muscles of Japanese quails

Indications	Gender		Significance of differences
	female ♀	male ♂	
Carcass weight (g)	179.8±10.28	163.0±15.59	NS*
pH_{24}	5.71±0.08	5.70±0.10	NS
Color parameters			
L*	33.20±2.08	32.02±2.54	*
a*	11.40±0.96	12.65±1.17	*
b*	8.95±0.82	9.05±0.96	NS
Cooking loss (%)	35.20±3.86	34.11±2.67	*

Notes: NS - the difference is not significant; *- the difference is determined at P<0.05.

Previous research has shown that the quality and composition of quail meat is influenced by many factors, such as genotype of birds (Genchev et al., 2005; Alkan et al., 2010), divergent selection (Maiorano et al., 2009), feeding (Gardzielewska et al., 2005), sex (Genchev et al., 2008), age (Tserveni-Gousi & Yannakopoulos, 1986), and stress (González et al., 2007). Table 2 shows the chemical composition of breast muscles of Japanese quails. The meat of the quails analyzed contained 71.0-71.49% water. According to other authors, quail breast and leg meat can contain 71 to 74% water (Hamm et al., 1982; Maron-Fuenmayor et al., 2008). Particularly valuable characteristics of quail meat include its high content of protein, essential vitamins and fatty acids. The protein content of quail breast meat ranged from 24.26% (females) to 24.70% (males). This is a higher result compared to studies done by other researchers who found the protein content of quail meat at 17-23% (Hamm et al., 1982; Maron-Fuenmayor et al., 2008). The fat content of the meat of the quails analyzed ranged from 2.70 to 2.79%. Genchev et al., (2008) found 2.5% fat in the breast meat of Japanese quails, while in studies by other authors the fat content ranges from 2-8% (Hamm et al., 1982; Maron-Fuenmayor et al., 2008). Khalifa et al. (2016) showed that meat from older quails (8 months old) was characterized by higher caloric content compared to meat from 6-week-old quails. Ionita et al. (2011) showed that quail meat had lower caloric content in comparison to the chicken and duck meat. Quail meat, due to its low fat content, is one of the low-calorie products, so this type of meat is increasingly popular among consumers (Ikhlas et al., 2011).

Table 2. Chemical composition breast muscles of Japanese quails

Parameter	Gender		Significance of differences
	female ♀	male ♂	
Total solids (%)	28.51±0.35	29.00±0.31	NS
Protein (%)	24.26±0.48	24.70±0.39	NS
Fat (%)	2.70±0.32	2.79±0.38	NS
Ash (%)	1.24±0.26	1.26±0.22	NS
Carbohydrates (%)	0.31±0.08	0.25±0.07	NS
Caloric value			
kcal/100 g	126±0.52	125±0.24	NS
kJ/100 g	517±6.2	529±3.7	NS

Notes: NS - the difference is not significant.

The composition of fatty acids in the quail meat is presented in Table 3. The fat content of Japanese quail breast meat was 2.70-2.79% and was dominated by four fatty acids: oleic (C18:1), palmitic (C16:0), linoleic (C18:2) and stearic (C18:0), which accounted for 86.6% of all fatty acids. Similar results were obtained by Genchev et al. (2008), Bonos et al. (2010), Sartowska et al. (2014) and Gecgel et al. (2015). According to Gecgel et al. (2015), Japanese quail meat can be included in a preventive diet for heart disease due to its high C18:1 content.

An important indicator of the health-promoting properties of a given fat is the ratio of PUFA to SFA, which according to World Health Organization (WHO) (2014) recommendations should be above 0.4. In our study, this ratio was 0.59. An even more favorable PUFA to SFA ratio in the fat on Japanese quails of the Pharaon breed of 0.73 was found by Genchev et al. (2008). A high oleic acid (C18:1) content of 37% is also beneficial, as this acid has a beneficial effect on lowering blood cholesterol levels and reducing the risk of ischemic heart disease. On the other hand, the PUFA n6/n3 ratio in the meat of the quail analyzed in our study was not favorable to consumers, ranging from 22.76-22.89. No particularly significant differences were found between females and males in the fatty acid profile.

Table 3. The fatty acids profile breast muscles of Japanese quails

Fatty acids	Gender		Significance of differences
	Female ♀	Male ♂	
C10:0	0.043±0.014	0.039±0.015	NS
C12:0	0.079±0.017	0.081±0.004	NS
C14:0	0.788±0.142	0.832±0.079	NS
C14:1	0.134±0.018	0.135±0.015	NS
C15:0	0.249±0.043	0.240±0.042	NS
C16:0	23.54±0.541	23.604±0.229	NS
C16:1 n9	0.520±0.012	0.527±0.028	NS
C16:1 n7	7.266±0.146	7.313±0.057	NS
C17:0	0.267±0.037	0.263±0.030	NS
C17:1	0.108±0.009	0.105±0.010	NS
C18:0	7.824±0.360	7.823±0.448	NS
C18:1 n-9	37.07±0.095	36.952±0.242	NS
C18:1 n-7	1.776±0.140	1.788±0.021	NS
C18:2 n-6	18.186±0.13	18.187±0.183	NS
C18:3 n-6	0.10±0.003	0.097±0.006	NS
C18:3 n-3	0.67±0.048	0.657±0.018	NS
CLA	0.10±0.006	0.099±0.004	NS
C20:0	0.17±0.018	0.17±0.008	NS
C20:1	0.54±0.029	0.53±0.029	NS
C20:2	0.09±0.022	0.09±0.016	NS
C20:3 n-6	0.02±0.005	0.02±0.004	NS
C20:4n-6	0.29±0.069	0.27±0.073	NS
C20:4n-3	0.005±0.001	0.006±0.001	NS
C20:5 n-3	0.056±0.005	0.059±0.006	NS
C22:4 n-6	0.009±0.001	0.009±0.001	NS
C22:5 n-6	0.014±0.004	0.016±0.002	NS
C22:5 n-3	0.064±0.006	0.066±0.004	NS
C22:6 n-3	0.027±0.008	0.026±0.004	NS
Other	0.01±0.001	0.005±0.003	NS
SFA[1]	32.963±0.10	33.05±0.385	NS
UFA[2]	67.028±0.10	66.945±0.416	NS
MUFA[3]	47.45±0.131	47.346±0.285	NS
PUFA[4]	19.61±0.151	19.599±0.200	NS
PUFA n-6	18.61 ±0.18	18.602±0.203	NS
PUFA n-3	0.818±0.017	0.813±0.015	NS
PUFA n6/n3	22.76±0.663	22.895±0.341	NS
PUFA/SFA	0.595±0.006	0.593±0.012	NS
PUFA/MUFA	0.414±0.004	0.414±0.012	NS
UFA/SFA	2.034±0.009	2.026±0.036	NS

Notes: NS - the difference is not significant; [1]SFA - saturated fatty acids; [2]UFA - unsaturated fatty acids; [3]MUFA - monounsaturated fatty acids; [4]PUFA - polyunsaturated fatty acids.

Table 4 shows the profile of amino acids in the breast muscle of the analyzed quails.

Table 4. The amino acids profile breast muscles of Japanese quails

Amino acids	Gender		Significance of differences
	Female ♀	Male ♂	
Essential AA			
Lysine	5.95±0.92	6.71±0.29	*
Methionine	3.81±0.62	3.35±0.79	NS
Isoleucine	7.40±0.95	7.76±0.84	NS
Leucine	8.99±0.40	8.95±0.23	NS
Phenylalanine	5.10±0.13	4.64±0.42	*
Threonine	5.11±0.35	4.80±0.68	NS
Valine	5.48±0.21	5.46±0.08	NS
Cysteine	0.93±0.16	0.74±0.13	NS
Tyrosine	4.07±0.23	3.89±0.60	NS
Total EAA	46.82±0.64	46.31±1.31	NS
Non-essential AA			
Histidine	4.44±0.03	3.98±0.27	*
Arginine	7.70±0.34	7.24±0.45	NS
Glutamic acids	12.85±0.11	13.67±0.66	NS
Glycine	5.80±0.29	5.76±0.27	NS
Serine	4.25±0.24	4.37±0.34	NS
Alanine	5.59±0.27	5.70±0.26	NS
Proline	4.24±0.30	3.82±0.20	*
Asparagine acids	8.30±0.21	9.13±0.49	*
Total non-essential AA	53.18±0.64	53.69±1.31	NS
Protein content	24,26±0,48	24,70±0,39	NS
Ratio nonessential/essential	1.14±0.029	1.16±0.061	NS
Ratio essential/nonessential	0.88±0.023	0.86±0.046	NS
Protein/essential	0.519±0.015	0.534±0.012	NS

Notes: NS - the difference is not significant; *- the difference is determined at P<0.05.

Nonessential amino acids predominated in the meat of the analyzed quails (53.18-53-69%); however, the essential/nonessential ratio ranged from 0.86 (males) to 0.88 (females). This meat was very tasty rich in essential amino acids that constituted approximately 46.31-46.82% of the meat protein. In a study by Khalifa et al. (2016), essential amino acids constituted about 41% of meat protein, and the essential/nonessential ratio was 0.60-0.63. Similar results are reported by Uherova et al. (1992). Glutamic acid and Asparagine acids predominated in the protein of the quail meat analyzed, which is consistent with the results of Genchev et al. (2008), Khalifa et al. (2016), Nasr et al. (2017). In our study, the meat protein of males contained more of these amino acids compared to that of females. Genchev et al. (2004) showed that the presence in quail meat of the limiting amino acids, protein, (methionine and lysine) accounts for about 11.8% of the total protein content of the product. In our study, the proportion of methionine and lysine in the meat of females accounted for 9.76% and in the meat of males for 10.06% of the total protein content of the product.

Quail meat owes its tenderness to thin muscle fibers Walasik et al. (2006). In addition, these authors found a lower intensity of pathological changes in muscle fibers, which can be explained by the high number of red fibers with oxidative metabolism.

Quail muscles are morphologically similar to the pectoral muscles of aquatic poultry in which relatively low intensity of pathological changes is found. Costăchescu et al. (2018) found no differences in the chemical composition of the meat of young female and male quail, while the meat of older males had a statistically significantly lower fat content compared to females. In addition, these authors showed that the pectoral muscles of female quails were characterized by lower cutting power compared to males. Quail meat is one of the products with low cholesterol content. Maiorano et al. (2011) reported a cholesterol level of pectoralis muscle in quail to vary from 23.57 to 37.20 mg. 100 g^{-1}, which was lower than the cholesterol content found by Maiorano et al. (2009) in the breast muscle of 35 day old Japanese quail (ranking from 27.83 to 43.38 mg.100 g^{-1}). Genchev et al. (2008) observed that the cholesterol content in quail carcass was 0.097 and 0.094 g.100 g^{-1} for males and females, respectively. Pavelková et al. (2020) observed differences between females and males in cholesterol content only in the pectoral muscles (females - 0.86 g.100 g^{-1}, males - 0.72 g.100 g^{-1}).

CONCLUSIONS

The chemical composition of quail meat, especially the high protein content and low fat content, make this meat characterized by a low caloric value. Quail meat is considered a good source of essential amino acids and fatty acids mainly from oleic, linoleic, palmitic and stearic acids. The meat of males was characterized by a higher content of lysine and glutamic acid and a lower content of histidine, arginine, tyrosine and methionine compared to the meat of females. The gender of quails had no significant impact on meat quality parameters.

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NATURAL INHIBITORS IN SOUS VIDE COOKING - A CRITICAL REVIEW

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Abstract

Sous vide (SV), a culinary technique that involves vacuum sealing food and cooking it at precise temperatures, has been celebrated for improving food's texture, flavor, and nutritional content. Integrating natural inhibitors is a crucial strategy for enhancing food safety, shelf life, and health benefits by fighting microbial growth. This review examines the combined effects of SV and natural inhibitors on various food types, including meats, seafood, and vegetables. It highlights significant progress in food safety, with natural additives helping to reduce pathogen resistance and curb bacterial spread, thus improving the overall safety and sensory appeal of meals. Moreover, it discusses the challenges and opportunities in refining the use of natural inhibitors in SV cooking, such as achieving the right balance between flavor and microbial management. The adoption of SV varies globally, reflecting its adaptability in different culinary traditions. The review suggests future research avenues, like exploring new natural inhibitors, cooking conditions, and their effects on consumer health, emphasizing SV's role in modernizing cooking practices to meet demands for quality, and safety.

***Key words**: culinary innovation, food safety, natural inhibitors, sensory quality, sous-vide.*

INTRODUCTION

In the evolving landscape of culinary innovation, SV cooking emerges as a paragon of modern culinary technique, blending the precision of scientific methods with the traditional artistry of cooking. This technique, denoted as "under vacuum" in French, entails vacuum-sealing food and cooking it in a water bath at carefully regulated temperatures, celebrated for its ability to maintain the food's integrity and amplify its flavors (Avató et al., 2022). Initially buoyed by the collaborative efforts of American and French chefs to enhance cooking consistency and quality, SV cooking has transitioned from a niche method in gourmet kitchens to a globally acknowledged culinary practice, marking a significant shift in food preparation approaches (Toon, 2018).

The hallmark of SV cooking lies in its distinctive temperature control and vacuum sealing method, offering unparalleled control over the texture, flavor, and nutritional value of food (Berdigaliuly et al., 2022; Song et al., 2023). This innovative cooking method enhances the sensory quality of culinary creations and introduces a novel way to incorporate natural inhibitors into food products, advancing food safety, extending shelf life, and improving nutritional content (Zakrzewski et al., 2023). The integration of natural inhibitors, such as oregano and citric acid, has shown potential in increasing the thermal sensitivity of bacteria in salmon (Dogruyol et al., 2020), while thyme and rosemary essential oils have showcased antimicrobial effects against *Listeria monocytogenes* in SV turkey and rainbow trout (Amoroso et al., 2019; Kačániová et al., 2021).

SV cooking's efficacy in preserving the nutritional and sensory qualities of food is robustly supported by literature. Its benefits extend to enhancing shelf life, a critical consideration in today's market that demands convenience without quality compromise. By employing low-temperature cooking, SV prevents the degradation of vital nutrients and bioactive compounds, enhancing food product longevity (Czarnowska-Kujawska et al., 2023). Further studies, such as those by Kaya et al. (2022), underscore the method's role in inhibiting bacterial growth and extending the shelf life of various food products.

The incorporation of natural inhibitors in SV cooking not only enhances food safety by preventing microbial growth but also enriches food with health-beneficial compounds. This synergy between SV cooking and natural inhibitors meets modern consumer preferences for food products that deliver health benefits beyond basic nutrition. For instance, the use of sage has been shown to improve food's oxidative stability and sensory properties, highlighting the method's dual function (Çetinkaya, 2020; Onyeaka et al., 2022).

As the market landscape evolves, a noticeable shift towards food products reflecting health, sustainability, and convenience values is evident. SV cooking, especially when coupled with natural inhibitors, adeptly responds to this trend, offering solutions that cater to contemporary consumer needs. This method extends shelf life, ensures food safety, and enhances nutritional quality, heralding a new era in culinary practices and signifying a transformative shift towards food innovation (Toon, 2018; Pongsetkul et al., 2023).

The current literature and emerging trends provide comprehensive insights into optimizing cooking conditions and identifying new natural inhibitors, guiding innovative culinary practices that prioritize health and sustainability. This review delves into the complex interplay between SV cooking and natural inhibitors, highlighting their combined impact on food safety, nutrition, and sensory qualities, taking into account current studies, future outlook of the development of this novel technology.

MATERIALS AND METHODS

This review article employs a comprehensive literature survey aimed at elucidating the interplay between SV cooking techniques and the integration of natural inhibitors for enhancing food safety, nutritional quality, and sensory attributes. The methodology followed to compile and analyse the current body of knowledge in this domain involved several systematic steps outlined below.

An extensive literature search was conducted across multiple scientific databases, including Google Scholar, PubMed, ScienceDirect, and Wiley Online Library, to gather relevant research articles, reviews, and case studies published up to 2024. The search strategy incorporated a combination of keywords related to "sous vide cooking", "natural inhibitors", "food safety", "sensory quality", and "culinary innovation" among others.

The inclusion criteria for the selection of articles were:

- Peer-reviewed articles and reviews published in English.
- Studies that specifically address the use of SV cooking techniques and/or the incorporation of natural inhibitors (e.g., essential oils, grape seed extract, citric acid).
- Research that provides empirical data on the effects of SV cooking and natural inhibitors on the microbial safety, nutritional content, and sensory properties of food products.
- Articles that discuss the commercial application, consumer perception, and regulatory considerations of SV cooking with natural inhibitors.

Exclusion criteria eliminated studies that:

- Did not directly relate to SV cooking or the use of natural inhibitors.
- Lacked empirical data or provided insufficient detail on methodology and results.
- Were published in languages other than English without an available English translation.

Relevant information was extracted from each selected article, including the year of publication, authors, study objective, methodology (SV conditions, types of natural inhibitors used, food matrix involved), key findings, and conclusions. This data facilitated a comprehensive analysis of the state-of-the-art in SV cooking with natural inhibitors, focusing on their impact on food safety, shelf life, nutritional quality, and sensory attributes.

Special attention was given to studies that reported innovative applications of natural inhibitors in SV cooking, documented challenges in integrating these substances into culinary practices, or offered insights into

future research directions and commercial potential.
The extracted data were synthesized to highlight emerging trends, consensus, and discrepancies within the reviewed literature.
This methodology underscores our commitment to rigorously reviewing and interpreting current research on SV cooking and natural inhibitors, thereby offering valuable insights into innovative culinary practices that prioritize health, safety, and sustainability.

RESULTS AND DISCUSSIONS

Use of inhibitors in SV processing

Within the scope of sous SV cooking, the strategic incorporation of natural inhibitors, such as grape seed extract and a spectrum of essential oils including oregano, rosemary, coriander, laurel, and sage, embodies a holistic approach to culinary arts. This methodology not only prioritizes the enhancement of food safety by mitigating pathogen resilience and controlling microbial proliferation but also enriches the sensory profile of dishes, marrying the essence of natural flavours with the principles of modern culinary safety.
When coming to the essential oil impact of the SV processed matrix, several aspects have been addressed in different studies, targeting the bacterial growth, preservation, and shelf life (Table 1).

Table 1. Essential oils and inhibitors in SV cooking

Inhibitor	Matrix	Key Finding	Autor
Grape Seed Extract	Ground Beef	Reduced bacterial heat resistance; enhanced safety	Cosansu et al., 2019
Grape Seed Extract	Doner Kebabs	0,5-1% addition lead to reduced bacterial heat resistance, energy conservation and enhanced quality	Haskaraca et al., 2019
Grape Seed Extract	Fresh Strawberries	1% addition lead to shelf life of up to 30 days	Duran et al., 2016
Grape Seed Extract	Roasted Chicken	0.2-1% addition lead to lower bacterial count on treated meat	Guo et al., 2020
Grape Seed Extract (GSE)	Refrigerated salmon	0,8% addition lowered *Pseudomonas spp*. on the 7th day of storage compared to control without GSE	Alves et al., 2018
Oregano Essential Oil	Potatoes	Controlled bacterial growth; enhanced flavor and shelf life	Zavadlav et al., 2020
Rosemary Essential Oil	Fresh cut Potatoe	Positive effect on texture, and limited the growth of mesophilic bacteria and *Enterobacteriaceae* over the storage period	Rizzo et al., 2018
Rosemary Essential Oil	Tilapia	Effective in preservation and extending shelf life	Zavadlav et al., 2020
Rosemary Essential Oil	Cook-Chill Beef	Reduction and antimicrobial effect against *L. monocytogenes*, extended shelf life	Gouveia et al., 2017
Coriander Essential Oil	Rainbow Trout	Effective against *L. monocytogenes*	Öztürk et al., 2021
Coriander Seed Extract	Ground lamb	The appearance, odor, and overall acceptability were better than the control, and reduction of *L. innocua*	Omidi-Mirzaei et al., 2021
Laurel Essential Oil	Rainbow Trout	Effective in vitro against *L. monocytogenes*	Öztürk et al., 2021
Laurel Essential Oil	Sea Bass	Extended the shelf life and enhanced flavor profile	Abouel-Yazeed et al., 2015
Laurel Essential Oil	European Sea Bass	Extended shelf life but there was a diminishing in quality	Bolat et al., 2019
Sage Essential Oil	Cooked Fish Fillet	Extended shelf life, decreased lipid oxidation	Çetinkaya et al., 2020
Sage Essential Oil	Beef Tenderloin	Suggested reduction in *L. monocytogenes* survival	Gál et al., 2023
Citric Acid	Mediterranean Mussels	Enhanced microbiological quality, reduced spoilage and lipid oxidation	Russo et al., 2023
Lime Juice	Chicken Breast	It prevented pink discoloration for up to 14 days	Hong et al., 2016

Studies like those of Cosansu et al. (2019) highlight the significant improvement in food safety these natural inhibitors bring, effectively reducing the heat resistance of pathogens and controlling bacterial growth, thereby extending the shelf life of perishable items like seafood. However, this approach is not without challenges; precisely calibrating the amount of each inhibitor to avoid overwhelming the natural flavours of the food can be a delicate balance. Overuse might lead to overpowering flavours or even ineffective pathogen control, and the cost or availability of high-quality natural additives could pose practical challenges, especially in commercial settings.

Plant extracts

In the field of food safety, the usage of ***grape seed extract*** (GSE) in ground beef offers significant advancements by markedly diminishing the heat resistance of *C. perfringens*, thereby enhancing food safety standards. Cosansu et al. (2019) highlight GSE's effectiveness, illustrating its role in substantially reducing pathogen survival rates during SV processing. This underscores GSE's potential as a formidable natural additive for augmenting both the quality and safety of SV meats. Complementarily, Haskaraca et al. (2019) further validate GSE's utility by demonstrating its impact when added at concentrations of 0.5-1% to vacuum-packaged, cook-in-bag döner kebabs. This inclusion notably heightens the heat sensitivity of *L. monocytogenes*, with Weibull model analysis revealing an increased susceptibility of the pathogen to thermal processing (Duru et al., 2020). Such insights present GSE as an efficacious measure for the food industry to heighten SV processed döner kebabs' safety against *L. monocytogenes*, thus assuring consumer safety, maintaining product integrity, and contributing towards energy efficiency.

The implications of leveraging GSE within SV culinary methods are particularly pronounced in commercial contexts, where ensuring food safety is paramount. The natural origin of GSE aligns seamlessly with the growing consumer predilection for clean-label ingredients, positioning it as an invaluable asset to the SV cooking process (Weslie et al., 2021). By integrating GSE, not only does it bolster food safety mechanisms, but it also resonates with consumer demands for natural and safe food solutions, thereby enhancing the appeal and acceptance of SV cooking methodologies in the broader food industry landscape.

Adding to the significance of GSE in food safety, recent studies have extended its application beyond meat products. The utilization of antimicrobial agents such as nisin, natamycin, pomegranate, and GSE in chitosan coatings has proven effective in extending the shelf life of fresh strawberries by preserving their quality and inhibiting microbial growth (Duran et al., 2016). Similarly, the development of chitosan films with GSE and carvacrol microcapsules has demonstrated positive effects on the shelf life of refrigerated salmon by maintaining quality parameters and reducing microbial counts, indicating the broad applicability of GSE across different food sectors (Alves et al., 2018). Furthermore, a study on roasted chicken has shown that a 0.5% GSE solution, combined with modified atmosphere packaging, significantly reduces microbial growth and lipid oxidation, thereby extending shelf life and maintaining product quality (Guo et al., 2020).

These findings collectively highlight the potential of GSE as a versatile and effective natural preservative that can be adapted across various food processing methods to ensure food safety and extend shelf life, aligning with consumer preferences for cleaner label products. The integration of GSE into food safety protocols across different food categories not only underscores its efficacy in enhancing food quality but also its importance in advancing food preservation technologies to meet evolving consumer demands and industry standards.

Essential oils

Rizzo et al., (2018) conducted a study that demonstrated the efficacy of integrating 0.5% ***rosemary essential oil*** in SV cooking (105°C for 15 min) in preserving the nutritional profile of potato slices during storage. Their findings highlighted a notable increment in the ascorbic acid content (4.3 mg/100 g) by the eleventh day, alongside a decrease in total phenolic content (TPC) by 11.53% and antioxidant activity by 48%. In a subsequent investigation, Rizzo et al. (2018) explored the impact of SV cooking (121°C for 30 min) with the addition of 0.5% rosemary essential oil (REO) on the quality of fresh-cut potatoes over storage. This study observed a significant reduction in ascorbic acid levels by 77% on the eleventh day compared to the initial values, with total phenolics and antioxidant activity experiencing around a 35% decline. The research attributed these effects to the presence of monoterpenic hydrocarbons, oxygenated monoterpenes, and sesquiterpene hydrocarbons in REO, known for their antioxidant and antimicrobial properties, thereby aiding in the maintenance of quality during storage.

Rosemary essential oil's application in tilapia has demonstrated efficacy in both preserving and extending the shelf life of the fillets, as evidenced by Zavadlav et al. (2020). This dual benefit of preservation and flavor enhancement is particularly valuable for seafood, making rosemary oil a distinctive choice for food preservation. Moreover, the utilization of REO in SV fish preparation can offer significant advantages to the seafood industry, serving as a natural and flavor-enhancing method to prolong shelf life and maintain quality. In addition to its use in tilapia, rosemary's broad-spectrum antimicrobial effects, especially against *L. monocytogenes*, have been substantiated in beef processed through SV cook-chill (SVCC) technology. Gouveia et al. (2017) noted that rosemary essential oil, at its minimum inhibitory concentration, achieved a notable additional 2-log10 reduction in *L. monocytogenes* populations over 14 days at both 2°C and 8°C, compared to controls. This highlights rosemary's potential as a natural preservative, emphasizing the importance of adequate chilling storage to mitigate consumer risk.

The implications of incorporating rosemary essential oil in SV fish preparation extend considerably within the food industry. Its application is not only advantageous for seafood but also shows promise in enhancing food safety and shelf life across various segments of the food industry, given its efficacy against pathogens like *L. monocytogenes*. The findings from the study by Gouveia et al. (2017) further support the potential of rosemary essential oil as a formidable antimicrobial agent, indicating its broad applicability in improving food safety and shelf life. The ability of rosemary oil to significantly diminish pathogen populations while concurrently enhancing flavor offers a dual advantage, positioning rosemary essential oil as a valuable asset in the food processing and preservation toolkit of the seafood industry.

Further emphasizing the utility of essential oils in SV processing, Gal et al. (2023) demonstrated that incorporating 0.5% (*w*/*v*) REO into fresh-cut sliced potatoes significantly curbed the proliferation of mesophilic bacteria and *Enterobacteriaceae*. This addition not only improved sensory attributes, such as sour and floury tastes, but also stabilized the hue value over 12 days of storage, effectively preventing browning and preserving visual appeal (Luo et al., 2019).

The application of ***oregano essential oil*** (OEO) has been identified as an effective measure to control bacterial growth in minimally processed potatoes prepared via SV, highlighting its efficacy in enhancing food safety and flavor (Zavadlav et al., 2020).

The broader application of OEO in SV cooking merges food safety with sensory enhancement, showcasing its natural antibacterial properties and aromatic profile to elevate the appeal and quality of SV vegetables. This integration aligns with increasing consumer preference for clean, safe, and flavorful food options, marking essential oils as key contributors to the advancement of SV culinary practices.

Coriander essential oil's (CEO) application to ground lamb showcased its efficacy in curbing *Listeria innocua*, as revealed by da by Omidi-Mirzaei et al. (2021), underscoring its potential as a potent natural antimicrobial agent suitable for SV cooking. The study conducted delved into the preservative effects of CEO on ground lamb mutton. This investigation highlighted that CEO significantly impacts pH levels, moisture content, colour indices, and sensory attributes of lamb mutton inoculated with *Listeria innocua* over an 8-day storage period across different temperatures. Remarkably, the essential oil maintained the meat's quality attributes, particularly at lower temperatures, demonstrating enhanced pH stability, moisture retention, colour preservation, and sensory appeal compared to control samples. These outcomes indicate CEO's capability as an efficient natural preservative, capable of extending the shelf life and preserving the quality of lamb mutton under suitable storage conditions, thereby affirming its value in the food industry for improving the safety and quality of meat products through natural methodologies.

The effectiveness of CEO in combating *L. monocytogenes* emphasizes the critical role of essential oils in bolstering the microbiological safety of SV products, especially pertinent for fish, where pathogen control is imperative. The findings from Omidi-Mirzaei et al. (2021) lend further support to the viability of CEO as a formidable antimicrobial agent, notably against *L. monocytogenes*, in SV cook-chill beef products. This suggests that the antimicrobial efficacy of CEO transcends fish, extending to other SV processed foods, indicating its widespread applicability in enhancing food safety and shelf life. The capability of CEO to significantly reduce pathogen populations while also enhancing flavor presents a dual advantage, positioning CEO as a valuable asset in the food industry's arsenal for processing and preservation.

The utilization of ***laurel essential oil*** (LEO) in rainbow trout has demonstrated promising antimicrobial properties against *L. monocytogenes*, showcasing its potential as a natural inhibitor for enhancing both safety and flavor in SV cooking methods. The study conducted by da Silva et al. (2017) effectively highlights LEO's efficacy in vitro, positioning it as a valuable natural antimicrobial agent. This finding is complemented by the work of Abouel-Yazeedv et al. (2015), which explores the use of natural preservatives, including LEO, in extending the shelf-life of sea bass during cooled storage, underscoring the potential for natural preservatives in seafood preservation.

Bolat et al. (2019) investigated the effects of LEO on microbiological, chemical, and sensory changes in vacuum-packed SV European sea bass under chilled conditions. Their findings further validate the efficacy of LEO as a natural preservative, highlighting its ability to mitigate microbial growth and enhance the sensory attributes of SV seafood.

These studies collectively underscore the significance of LEO in the culinary and safety aspects of SV cooking, offering a natural solution to food safety concerns. By integrating LEO, the food industry can address modern consumer preferences for natural ingredients and traditional culinary techniques, enhancing the microbiological safety and overall quality of SV products, particularly in seafood, where pathogen control is paramount.

Sage essential oil's (SEO) application to beef tenderloin, as explored by Gal et al. (2023), has shown significant promise in reducing the survival of *L. monocytogenes*, highlighting its potential as a natural additive for enhancing meat safety through SV cooking. This aligns with further research by Çetinkaya (2020), which demonstrated sage's (*Salvia officinalis*, Linnaeus 1753) application to SV-cooked fish fillets, revealing significant antioxidant effects without compromising the nutritional and sensory properties of the fish. Sage essential oil not only maintained the fillets' quality by mitigating spoilage and lipid oxidation but also extended their shelf life and sensory acceptability by at least five days. These observations underscore sage's role as an effective natural antioxidant in SV-processed foods, providing a beneficial approach for food preservation that safeguards both sensory and nutritional values.

Moreover, the effectiveness of sage essential oil in combating *Salmonella enterica* during beef SV storage (Gál et al., 2023) and the impact of thyme essential oil in improving the quality and microbial safety of fresh-cut potatoes (Sarengaowa et al., 2023) underscore the wide-ranging benefits of essential oils in ensuring food safety while enriching sensory experiences in SV cooking.

Drawing upon the research by Gál et al. (2023), a case study highlights the use of sage essential oil as a natural inhibitor during the SV cooking of beef tenderloin. The study demonstrated sage essential oil's capacity to reduce the survival of *L. monocytogenes*, thereby enhancing meat safety. Furthermore, the addition of sage not only contributed to microbial safety but also improved the oxidative stability and sensory properties of the beef tenderloin, indicating an increase in shelf life and consumer appeal. This example illustrates the dual benefits of using natural inhibitors in SV cooking: ensuring food safety and enriching sensory quality, making it an attractive option for the meat industry.

The broader implications of SEO's effectiveness in reducing pathogen survival, particularly in beef tenderloin, point towards a promising avenue for natural food safety solutions within meat processing. Especially relevant for SV cooking, where the equilibrium between flavor and safety is crucial, SEO offers a natural method to address food safety concerns. This is particularly pertinent given the findings of Ismail et al. (2019), which suggest sage essential oil's potential in not only safeguarding against pathogens like *L. monocytogenes* but also in enhancing the overall quality of meat products through its antioxidant properties. Consequently, SEO emerges as a valuable asset for the food industry, advocating for its integration into meat processing and preservation strategies to achieve a balance of flavor, safety, and nutritional integrity.

In SV cooking, the strategic use of natural inhibitors and essential oils such as grape seed extract, oregano, rosemary, coriander, laurel, and sage essential oils, as well as citric acid, demonstrates significant synergistic effects. These combinations not only enhance the sensory qualities of foods like ground beef, potatoes, tilapia, and rainbow trout but also markedly improve food safety and extend shelf life. Studies by Cosansu et al. (2019), Zavadlav et al. (2020), da Silva et al. (2017), Ismail et al. (2019), and Russo et al. (2023) highlight these effects, showcasing how these inhibitors work in tandem to reduce bacterial resistance, control microbial growth, and impart nuanced flavours. The interplay between these natural additives and the food matrices creates products that are not only safe and longer-lasting but also more enjoyable in terms of taste, aligning with contemporary culinary trends towards natural, clean-label ingredients.

Organic acids

Russo et al. (2023) conducted an insightful study on the SV cooking of Mediterranean mussels (*Mytilus galloprovincialis*), where citric acid was used as a natural inhibitor. This approach not only aimed at ensuring food safety but also at assessing the quality of the mussels post-cooking. The findings revealed that incorporating citric acid into the SV cooking process significantly reduced spoilage and lipid oxidation, thereby enhancing the microbiological quality of the mussels. This case exemplifies how SV cooking, when coupled with citric acid, can serve as a powerful tool to maintain and even improve the quality of seafood, promoting a healthier and safer consumption experience.

Building on the innovative work of Russo et al. (2023), the study by Hong et al. (2016) further explores the realm of natural food preservation techniques, specifically focusing on the SV cooking of chicken. In this study, lime juice and citric acid were evaluated for their effectiveness in preventing pink discoloration and inhibiting bacterial growth in SV-cooked chicken breasts. The results demonstrated that both lime juice and citric acid effectively reduced pH levels, pink discoloration, lipid oxidation, and expressible drip while also enhancing microbiological safety without negatively impacting sensory qualities. These findings underscore the potential of natural additives like lime juice and citric acid in improving food safety and quality, aligning with consumer preferences for natural ingredients.

The convergence of findings from Russo et al. (2023) and Hong et al. (2016) illustrates a broader trend towards leveraging natural inhibitors to address food quality and safety challenges in different culinary contexts. This body of research validates the notion that natural additives can be successfully integrated into modern cooking techniques like SV cooking to deliver enhanced food quality, extend shelf life, and ensure safety, without compromising sensory characteristics. Thus, these studies collectively contribute valuable insights into the development of healthier, safer, and more sustainable food processing methods, emphasizing the importance of natural preservatives in the culinary and food science fields.

Generally, the experiments involved the use of only one natural inhibitor, and big source for exploration is given by the use of inhibitors’mixture. A ground-breaking study by Abouel-Yazeed et al. (2015) explored the effectiveness of using natural preservatives such as laurel and other botanical extracts to control the shelf-life of sea bass (*Dicentrarchus labrax*) during refrigerated storage. This investigation highlighted the synergistic potential of combining SV cooking with natural preservatives to not only extend the shelf life of seafood but also to enhance its flavor profile. By incorporating laurel essential oil in the SV packaging, the sea bass demonstrated significant improvements in microbiological stability and sensory attributes, including taste and aroma. This case study underscores the commercial viability of integrating natural preservatives in SV cooking processes, offering a sustainable method to preserve seafood with added flavor benefits.

Future research directions

The search for new natural inhibitors that can be integrated into SV cooking processes remains a promising area of research. With an ever-growing database of plant-based compounds exhibiting antimicrobial and antioxidant properties, future studies should focus on evaluating the efficacy of less common herbs, spices, and plant extracts. Research akin to the studies by Kačániová et al. (2021) and Gál et al. (2023) could expand to explore the potential of underutilized botanicals in enhancing food safety, sensory qualities, and nutritional value of SV-cooked foods.

Given the diversity of food types and their unique characteristics, there's a significant need to optimize SV cooking parameters (e.g., temperature, time, vacuum level) for different food matrices. Future research could build on the foundational work by Czarnowska-Kujawska et al. (2023) and Pongsetkul et al. (2023), investigating how varying SV conditions affect the interaction between food products and natural inhibitors, particularly focusing on the retention of nutritional and sensory attributes.

While the immediate benefits of SV cooking with natural inhibitors on food safety and quality are well documented, there's a gap in understanding the long-term health impacts of consuming such foods. Future studies should aim to assess the cumulative effects of regular consumption of SV-cooked foods with natural inhibitors on human health, similar to the perspective offered by Onyeaka et al. (2022). This research could provide critical insights into the dietary benefits or potential risks associated with these culinary practices.

Technological advancements in SV cooking equipment could further enhance the efficacy of natural inhibitors. Future research could explore the development of specialized SV devices that can better regulate the diffusion of natural inhibitors into the food matrix, ensuring uniform flavor and safety profiles. Studies focusing on equipment innovation, akin to the investigation of cooking conditions by Luo et al. (2019), could lead to more precise and efficient SV cooking methodologies.

The integration of natural inhibitors in SV cooking also presents an opportunity to investigate the economic and environmental sustainability of this culinary technique. Future research, building on the life cycle assessment model discussed by Avató et al. (2022), should explore the cost-effectiveness and ecological impact of sourcing and applying natural inhibitors in commercial SV cooking. This includes assessing the carbon footprint of producing these inhibitors and evaluating their role in reducing food waste through extended shelf life and improved safety.

Understanding consumer acceptance and market trends is crucial for the successful commercialization of SV cooking with natural inhibitors. Future studies should delve into consumer perceptions of SV-cooked foods enhanced with natural inhibitors, exploring preferences, willingness to pay, and perceived health benefits. This line of inquiry could follow the model of market landscape evaluations by Toon (2018), focusing specifically on how natural inhibitors influence consumer choices and acceptance in different culinary cultures.

The outlined future research directions aim to address the gaps in current knowledge and explore new horizons in the field of SV cooking with natural inhibitors. By focusing on these areas, researchers can contribute to the development of innovative culinary practices that prioritize health, sustainability, and sensory enjoyment. Collaboration between food scientists, chefs, and industry stakeholders will be key in advancing this field, ensuring that SV cooking with natural inhibitors remains at the forefront of culinary innovation and consumer preferences.

CONCLUSIONS

The integration of (SV) cooking with natural inhibitors represents a forward-looking approach that marries culinary innovation with food safety, nutritional enhancement, and sensory quality. This review has emphasized the transformative potential of SV cooking in leveraging natural inhibitors like essential oils and grape seed extract, not only to elevate the gastronomic experience but also to address contemporary consumer demands for healthful, sustainable, and flavorful food options. Future research directions, including the exploration of new natural inhibitors, optimization of SV parameters for diverse food matrices, and assessment of long-term health impacts, underscore the dynamic potential for growth and innovation within this field. As this culinary technique continues to evolve, it holds promise for revolutionizing food preparation, consumption, and appreciation, heralding a new era in the gastronomic landscape that prioritizes health, sustainability, and culinary excellence

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NON-DAIRY YOGHURT ENRICHED WITH FUNCTIONAL PLANT-BASED INGREDIENTS - A REVIEW

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Abstract

The paradigm surrounding milk has shifted as consumer preferences evolve due to lactose intolerance awareness and the prevalence of cow's milk allergies. Consequently, plant-based milk substitutes have gained popularity. This study offers a comprehensive examination of the physicochemical, rheological and sensory attributes of various functional plant-based yoghurts, coconut yoghurt with tapioca, fruits and soy yoghurt with red fruits, mango, lemon grass, etc. The study reveals the potential of these products to fulfil consumer demands for both sensory satisfaction and nutritional benefits. Furthermore, it identifies avenues for further refinement and innovation in product development and formulation, thereby providing valuable insights for both industry stakeholders and consumers seeking viable dairy alternatives. This study underscores the growing importance of plant-based options in meeting diverse dietary needs and preferences in today's evolving food scene. Moreover, it emphasizes the necessity of transitioning to a more plant-based diet to address climate change, reduce environmental impacts, and improve health.

***Key words**: functional, non-dairy yoghurt, plant-based.*

INTRODUCTION

Yoghurt is widely acknowledged as a delicious and nutritious food, rich in essential vitamins and minerals. For individuals prioritizing sustainability in their diets, those with lactose intolerance, or those opting for non-dairy alternatives like vegans, lactose-free plant-based yoghurt becomes a preferred choice.

Typically, these individuals, as well as those eager to explore new food options, find the most satisfaction when the plant-based yoghurt alternative closely resembles the appearance and texture of traditional dairy yoghurt.

The increasing demand for non-dairy yoghurts, along with the growing interest in plant-based milk and meat alternatives, has spurred the development of a plant-based food industry valued at USD 5 billion, reshaping the culinary landscape in America.

Over the past 15 years, there has been a remarkable 300% surge in the number of Americans embracing plant-based diets (International, 2024).

The surge in interest for alternative foods is on the rise, spurred by the imperative to sustainably nourish the expanding global population. Companies and consumers alike are increasingly focused on sourcing foods that are both eco-friendly and nutritious. While plant-based dairy substitutes have been consumed for generations in various cultures, there's a renewed curiosity driving their market expansion. Time-honoured options like Spanish Horchata and Asian soya milk stand alongside modern favourites such as soy, almond, and rice-based milk substitutes (Mäkinen et al., 2016).

In recent years, scientific research has emphasized the significant positive impact of shifting towards plant-based diets on ecosystems. This dietary change can reduce environmental impact, preserve biodiversity, and mitigate climate change (Westhoek et al., 2014).

Several studies analysing the health effects of plant-based diets indicate the potential for significant benefits (Zhong et al., 2021). Dairy is facing significant competition from these plant-based alternatives, particularly in Western countries, where the demand for such products is escalating (International, 2020). The development of non-dairy yoghurt holds significant importance in meeting the nutritional needs of both elderly individuals and those seeking energy-dense, protein-enriched food options, addressing a gap in the market. Over recent years, soybean, oat, and coconut have gained recognition as functional foods due to their protein, dietary fibre, mineral, antioxidant, vitamin, and energy content (Inglett et al., 2003). Soybean and oats particularly serve as cost-effective protein sources, catering to vegetarians, vegans, and individuals with limited access to meat and dairy (Oyeniyi et al., 2014). Non-dairy yoghurt, containing unsaturated fatty acids, aids in reducing the risk of cardiovascular diseases and proves beneficial for lactose intolerant individuals. Furthermore, non-dairy yoghurts boast high levels of nutrients and minerals, acting as symbiotic foods crucial for gut health and immunity enhancement (Osundahunsi et al., 2007). Soy milk, oat milk, and coconut milk serve as viable substitutes for animal milk in dairy product manufacturing. Fermentation of milk has been reported to diminish antinutritional factors and enhance mineral bioavailability (Adeyemo et al., 2013). Yoghurt, whether dairy or non-dairy, serves as a carrier for probiotics, rich in protein, magnesium, potassium, fat, and vitamins (Lourens-Hattingh et al., 2001). Non-dairy yoghurts typically utilize low-fat milk, coagulating to a custard-like consistency, and contain cultures like *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (Potter et al., 2012). Soy yoghurt fermentation primarily involves friendly bacteria, notably *L. bulgaricus* and *S. thermophilus*, utilizing sugars such as stachyose and raffinose instead of lactose. Soy protein offers nutrients equivalent to those found in meat and eggs, essential for human health (Oyeniyi et al., 2014). Extensive research has been conducted on probiotics due to their potential health benefits. According to definitions provided by the Food and Agriculture Organization and the World Health Organization, probiotics are live microorganisms that, when consumed in adequate amounts, enhance the health of the individual (Hill et al., 2014).

For probiotics to be effective, they must possess certain essential characteristics. These include robust stability during storage and distribution, non-pathogenic and non-toxic properties, sustainability within the host's body, ability to adhere effectively, resistance to low pH and bile salts in the gastrointestinal tract, and positive activity that promotes beneficial effects on the host, such as enhanced immunity (Shori et al., 2021).

To ensure the health benefits of fermented plant-based milk products, it's essential for probiotics to meet the minimum level requirement of probiotic bacteria ranging between 10^6 and 10^7 colony-forming units (CFU) per millilitre until the expiration date (Shori et al., 2018).

MATERIALS AND METHODS

For this review, we conducted a comprehensive literature search across multiple databases including Web of Science, PubMed, Google Scholar, and ScienceDirect, using the key words "yoghurt", "functional food", "non-dairy food", and "plant-based foods" within the timeframe of 2011 to January 2024. After removing duplicates, we focused exclusively on studies pertaining to non-dairy food products. Following this initial screening, articles meeting the inclusion criteria were selected based on their titles and abstracts, with a broad interpretation applied.

The inclusion criteria for article selection were as follows:

- Peer-reviewed articles and reviews published in English;
- Studies specifically addressing non-dairy functional fermented products (e.g., soy yoghurt with lemon grass);
- Research providing empirical data on the functional properties, as well as other important physicochemical and microbiological properties, of plant-based yoghurt;
- Articles discussing the significance of non-dairy products.

Exclusion criteria were applied to eliminate studies that:

- Did not directly pertain to non-dairy products;
- Lacked empirical data or failed to provide sufficient detail on methodology and results;

- Were published in languages other than English without an available English translation.

Selected articles underwent a thorough evaluation, with relevant information such as publication year, authors, study objectives, methodology, key findings, and conclusions extracted. This data enabled a comprehensive analysis of non-dairy plant-based functional yoghurts from various perspectives, including shelf life, nutritional quality, antioxidant activity, and sensory attribute. Special attention was given to studies showcasing innovative uses of functional ingredients in plant-based yoghurt production, along with those offering insights into future research and commercial potential.

The data gathered was carefully synthesized to highlight emerging trends, areas of agreement, and discrepancies within the literature we reviewed. Our rigorous methodology demonstrates our commitment to thoroughly examining and interpreting current research on non-dairy plant-based functional yoghurt,

RESULTS AND DISCUSSIONS

1. Technology of plant-based yoghurt

Currently, plant-based yoghurt products utilize the conventional yoghurt production method, which involves fermenting plant-based milks (Kizer et al., 2023). To prepare a cow milk yoghurt alternative, the milk samples were pasteurized at an optimum temperature of 72°C for 20 min by a double boiling method to avoid gelatinization. This method uses the steam from the simmering water to warm the milk in the bowl gently, with indirect heat. Then, the milk was cooled to 45°C. The starter cultures (*L. bulgaricus* and *S. thermophilus*) were added as 0.4% of the milk mixture weight. After inoculation, 10% of sucrose was added to the milk mixture to optimize the growth of lactic acid bacteria. To strengthen the gel network of the yoghurt, corn starch was added (5%) at above 60°C, xanthan gum (0.15%) at above 70°C under continuous stirring, and pectin (0.75%) at above 25°C.

The milk was incubated at 41°C for 18 h to maintain the humidity and temperature in favourable conditions for the growth of microorganisms. The formed yoghurt was cooled to a room temperature of 27°C and stored in a refrigerator at 4°C for 1 h (Paul, 2020).

2. Plant-based raw material alternatives

Due to its important nutritional characteristics, such as calcium, high-quality proteins and an adequate level of isoflavones, which prevent bone degradation and have anticancer effects, the inclusion of plant based milk alternatives and its by-products in the diet is raising substantial interest (Pachekrepapol, 2021; Rahmatuzzaman, 2021).

The preparation of yoghurt consists of the lactic acid fermentation of milk by the action of starter bacteria (Salehi et al., 2021). Unfortunately, plant based yoghurt alternatives present texture and stability limitations compared to dairy yoghurts. The various textures of commercial plant based yoghurt alternatives could be caused by their reduced protein concentrations, and because these proteins do not coagulate as well as casein, gelling agents need to be added (Pachekrepapol et al., 2021). On the other hand, novel ingredients such as peas, lupins, oats, quinoa, and different type of fruits (strawberries, raspberry, blueberries) are also being assessed to improve the physicochemical characteristics of vegan yoghurt alternatives and the overall impression of consumers. For example, the physicochemical and sensory characteristics of soy yoghurt alternatives with lactic acid cultures (10^6 CFU/mL) containing 0.3% gelatine, strawberry were improved compared to the control sample (Salehi et al., 2021).

The adoption of plant-based milk, such as those derived from almonds, soy, and corn, as primary ingredients in yoghurt production has garnered significant interest in recent years. The food industry has responded by developing plant-based milk beverages to meet this demand. Almond milk, in particular, has gained popularity due to its protein content and water intake being comparable to that of bovine milk. Soy milk is another widely used plant-based milk, finding its way into various non-dairy products such as soy cheese, tofu, and soy yoghurt (Mäkinen et al., 2016). Almond milk stands out as a nutritious option due to its rich content of various essential nutrients. It contains significant amounts of vitamin E

(25.87 mg/100 g α-tocopherol), B-complex vitamins, proteins (16-23 g/100 g), monounsaturated fats (31-35 g/100 g) primarily consisting of oleic acid, dietary fibres (11-14 g/100 g), total phenolic compounds (260-350 mg/100 g), and various minerals. Despite almonds being relatively more expensive, almond milk has found its place among other plant-based milk substitutes in the market (Mäkinen et al., 2016).

On the other hand, coconut, known for its nutritional value, yields coconut milk which presents the highest fat content and the lowest protein content among non-dairy milk options. Notably, approximately 87% of the fat content in coconut milk is saturated, with lauric acid being the predominant component (44%), followed by caprylic and capric acids (13%). However, this high saturated fat content may potentially elevate harmful cholesterol levels (LDL) (Paul et al., 2020).

Cashew milk is rich in beneficial fats like monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs). With its oil content comprising 48%, it provides a significant source of these healthy fats. Oleic acid, a key monounsaturated fat, constitutes approximately 73.73% of the fat content. Additionally, cashew milk contains about 4.7% ash content, 19.8% protein, and 1.2% fibre, contributing to its status as a low-calorie beverage option. The ample presence of MUFAs and PUFAs, along with its relatively low calorie count, positions cashew milk as a nutritious alternative to traditional dairy milk and other plant-based milk varieties (Tamuno et al., 2019). Groundnut, commonly known as peanut, is an economical source of nutrients and is often utilized in the production of nut milk, which is considered akin to a complete food similar to cow's milk. Groundnut milk provides a substantial amount of carbohydrates, ranging from 49% to 63.5%, as well as protein content ranging from 4.5% to 7.4%. Additionally, it offers dietary fibre in the range of 3.2% to 4.4%, and notably contains negligible cholesterol content at 0.01%, in contrast to cow milk. Given its nutritional composition and affordability, groundnut milk serves as a viable and widely accepted alternative for the production of nut milk-based food items (Murevanhema et al., 2013).

Oats have been a staple food for centuries, valued for their nutritional content, particularly their high fibre content. Oat milk, derived from oats, is notable for its elevated levels of protein, carbohydrates, and dietary fibres compared to other plant-based milk alternatives. Research indicates that beta-glucan, a type of fibre found in oat milk, possesses cholesterol-lowering properties, making it beneficial for heart health (Ho et al., 2016).

Quinoa, characterized by its small round yellow seeds, is abundant in protein, starch, fibres, and essential fatty acids. This versatile grain has found applications not only in traditional dishes but also in the production of fermented alcoholic beverages. Additionally, quinoa remains relatively unexplored as a grain for the creation of plant-based foods, presenting opportunities for further culinary innovation and exploration (Srujana et al., 2019).

In the realm of plant-based yogurt production, the utilization of rennet derived from plant sources mirrors an emerging trend within the dairy industry. Innovatively, an array of fresh cheeses can now be obtained through coagulation with lettuce, echoing the trend in the dairy industry towards utilizing plant-based methods. This approach not only caters to the preferences of vegetarians but also offers nutritional benefits, as it allows for the incorporation of bioactive compounds from plant sources into the cheese-making process, similar to advancements seen in plant-based yogurt production (Nitu et al., 2022).

Therefore, among the most commonly encountered are coconut and soy yogurt, with various additives, described in the following section.

3. Functional coconut yoghurt

Coconut (*Cocos nucifera*) holds significant economic importance, particularly in Southeast Asian nations (Sethi et al., 2016). In recent times, considerable focus has been directed towards coconut milk as a viable alternative to dairy milk. With fat content ranging from 31% to 35% and protein content between 3.5% and 4.0%, coconut milk is recognized for its richness in essential amino acids, as well as its abundance in vital nutrients such as calcium, phosphorus, potassium, vitamin C, E, and B6, making it easily digestible (Góral et al., 2018). Coconut milk naturally forms an oil-in-water emulsion. This emulsion is stabilized by coconut proteins (globulin and albumin) and phosphor-lipids, which act as emulsifiers. These components adhere to the surface of coconut oil droplets, preventing phase separation (Lu et al., 2018).

Unlike allergies to tree nuts, coconut allergies are uncommon and are not directly associated with nut allergies (Anagnostou et al., 2017).

There are limited studies focusing on fermented coconut milk products. Many of these studies have included other protein sources, such as chickpea (Mesquita et al., 2020), which can raise the risk of allergenicity including additives which may not be in line with the rising demand for clean label products (Montemurro et al., 2021).

3.1. Coconut yoghurt with tapioca

Replacing cow milk with coconut milk in the production of yoghurt with both high consumer acceptance and optimal characteristics poses a significant and somewhat challenging issue. Often, stabilizers or thickeners are added to attain the desired texture and minimize the syneresis (Pachekrepapol et al., 2021).

The objective of the study conducted by Pachekrepapol (2021) is to create a yoghurt-like product using coconut milk while minimizing the use of additives. Initial investigations revealed that incorporating *tapioca starch* resulted in a smoother texture with reduced syneresis compared to other tested stabilizers such as pectin, xanthan gum, and corn starch. The study involved formulating samples with tapioca starch at concentrations of 0.5%, 1.0%, 1.5%, and 2.0% (w/w). These products were then assessed for pH variations and viability of lactic. The results are presented in Table 1.

3.2. Pineapple coconut yoghurt

In addition to its function as an aroma enhancer, pineapple can enhance the rheological properties of food items, particularly in terms of texture, mouthfeel, and colour. Moreover, pineapple serves as an additional source of dietary fibre, attributed to its high pectin content (García-Cano et al., 2019). The utilization of fortified pineapple puree (*Ananas comosus* (L.) Merr) in this study was chosen not only due to the widespread cultivation of pineapple but also because of its health-promoting properties. Pineapple is rich in beneficial compounds such as antioxidants, bromelain enzymes, phenolic compounds, and organic acids, as highlighted in studies by (Barzegar et al., 2023). The novelty of Parhusip's (2024) study lies in utilizing plant-based ingredients like coconut milk as the primary material for yoghurt production, aimed at decreasing reliance on animal-based food ingredients. Addressing the drawbacks of coconut milk, particularly its susceptibility to lipid oxidation, is achieved through the incorporation of pineapple puree, which serves as an inhibitor. The objective of Parhusip's study (2024) is to identify the optimal fermentation duration for coconut milk yoghurt, focusing on both physicochemical parameters (including pH, acidity, water holding capacity, viscosity, and fat content) and microbiological parameters (such as total plate count and total lactic acid bacteria). The rise in soluble solids, attributed to the addition of pineapple puree, enhances the water holding capacity and viscosity of yoghurt. The pectin content present in pineapple serves as a filler material in coconut milk yoghurt. Coconut milk yoghurt fortified with pineapple contains predominantly lauric acid, offering potential health benefits. Thus, employing coconut milk with pineapple fortification as plant-based ingredients for yoghurt presents promising prospects as a functional food alternative (Parhusip et al., 2024). Other results are presented in Table 1.

3.3. Strawberry coconut yoghurt

Enhancing the nutritional profile of plant-based milks involves incorporating beneficial micro-organisms that support the human microbiota, alongside fruit pulps to enhance nutrient content and flavour (Bedani et al., 2014).

The study of Mauro et al. (2022) aimed to create and analyse two coconut milk products fermented by *Lactobacillus reuteri* LR 92, with and without strawberry pulp. Additionally, it sought to determine the optimal concentration of guar and xanthan gum for product stability, assess the fatty acid profile, evaluate microorganism survival post-gastrointestinal simulation, and conduct sensory acceptance tests for the products. During sensory analysis, the overall acceptance scores for coconut milk products were notably high, averaging above 7.5 on a 9-point scale. Lauric acid emerged as the predominant fatty acid in the products. These findings indicate that fermented coconut milk products are well-received and can serve as effective carriers for *L. reuteri*, offering consumers functional fermented non-dairy options (Mauro, 2022). More results are included in Table 1.

Table 1. Coconut yoghurt with functional ingredients

Functional ingredient	Concentration of the functional ingredient	Proprieties	References
Tapioca	0.5%, 1.0%, 1.5%, 2.0% (w/w)	The pH levels of all samples decreased during 14-day storage period at 4 ◦C The syneresis level of all samples decreased with storage time The decrease in viscosity during shearing indicated shear-thinning behaviour of the samples. Yoghurt-like products made from coconut milk with 1.0, 1.5 and 2.0% tapioca starch received significantly higher scores for overall acceptability and flavour ($p < 0.05$)	(Pachekrepapol et al., 2021)
Pineapple puree	0%, 4%, 8%, 12%	pH : • Coconut milk unfortified 6.11±0.03 • Pineapple puree 3.90±0.01 • Pineapple puree fortification (4%) 5.89±0.04 • Pineapple puree fortification (8%) 5.66±0.03 • Pineapple puree fortification (12%) 5.49±0.06 Fat content 10.57-15.12±0.06 % Viscosity 2547- 3360±55.84 cP Total plate count 7.11-7.48±0.34 log cfu/g	(Parhusip et al., 2024)
Strawberry pulp	20% (w/v)	Moisture: 84.27b (± 0.04) % Proteins: 0.32a (± 0.01)% pH: 4.02c (± 0.03)% Ashes: 0.11a (± 0.05)% Lauric acid, C12:0 (SFA, MCFA): 41.78A ± 1.13 The fermented coconut products presented a satisfactory number of viable cells after gastrointestinal simulation.	(Mauro et al., 2022)

4. Functional soy yoghurt

Soybean milk serves as an alternative for those unable to consume cow's milk due to lactose intolerance or milk protein allergies. Derived from the *Glycine max* (L.) Merrill plant, soybean is rich in protein, making it a cost-effective protein source (Myagmardorj et al., 2018).

Among the ingredients used in the preparation of non-dairy functional yogurts, are vegetables such as mung beans, fruits in the form of powder or pulp, and aromatic plants in the form of essential oils.

4.1. Soy yoghurt enriched with legumes

Soy yoghurt enriched with mungbeans

Mungbeans, scientifically known as *Vigna radiata* (L.) R. Wilczek, boast abundant vitamins and minerals. Furthermore, they exhibit a higher carbohydrate content (approximately 62.3%) and lower fat content (approximately 1.9%) compared to soybeans. Soybeans, in contrast, contain around 33.9% carbohydrates and approximately 21% fat (Dahiya et al., 2015). Mungbean demonstrates a notably higher total phenolic content and antioxidant activity compared to soybean. Additionally, mungbean exhibits greater tyrosinase inhibition than other legume crops, potentially contributing to the prevention of type II diabetes (Yao et al., 2011). The elevated carbohydrate content in mungbean milk could lead to a reduction in pH following fermentation (An et al., 2024). This study conducted by An suggests that fermented mungbean milk holds promise as a food ingredient for creating milk substitutes when combined with soy milk. This combination has the potential to enhance the nutritional balance of dairy products. Additionally, it explores the feasibility of utilizing mungbean milk as a substrate for lactic acid bacteria (LAB) fermentation.

4.2. Soy yoghurt enriched with fruits

Soy yoghurt enriched with red dragon fruit

Among the fruits used to enhance non-dairy yogurts are dragon fruit, red fruit extract, mango pulp, and lemon juice. Soy milk, known for its rich content of high-quality plant protein, has been explored as a base for producing probiotic-enriched yoghurt, promoting digestive health (De et al., 2022). Thus, the replacement of red dragon fruit, renowned for its abundance of antioxidants and natural pigments, was aimed at enhancing both the antioxidant properties and aroma of soy yoghurt. The research of Marjan et al. (2023) delved into assessing the antioxidant activity and lactic acid bacteria levels in soy yoghurt infused with red dragon fruit. This analysis encompassed sensory assessment, proximate analysis, determination of antioxidant activity, and estimation of total Lactic Acid Bacteria (LAB). Among the various formulations tested, Formula 3, featuring a 35% substitution of red dragon fruit, emerged as the optimal choice. Proximate analysis revealed a water content of 92.75%, ash content of 0.23%, protein content of 0.48%, fat content of 3.08%, and carbohydrate content of 3.46% (Marjan et al., 2023).

Soy-based yoghurt enriched with red fruit extract

Red fruit extract (*Pandanus conoideus* Lam.) has been found to contain significant amounts of β-carotene and α-carotene, with levels of 130 µg and 1,980 µg per 100 g of sample, respectively. Additionally, it exhibits a high antioxidant content, particularly in terms of α-tocopherol, with a concentration of 21.20 mg per 100 g of sample (Surono et al., 2008). Given the notably high antioxidant content found in red fruit, which is recognized for its potential to combat free radicals, there's a compelling need to innovate processed food items by incorporating red fruit paste ingredients. This strategic utilization aims to yield food products of superior quality, not only in terms of their functional efficacy but also their sensory attributes (Tang'nga et al., 2019). The objective of Tang’nga’s research is to develop functional food products, specifically focusing on the predominance of soy milk yoghurt combined with red fruit paste. This innovative combination aims to offer an alternative to animal milk consumption while simultaneously providing antioxidant activity. Results about the pH, ash content, moisture and other are presented in Table 2.

Soy yoghurt with mango pulp

Incorporating protein-enriched yoghurt with fortified fruits presents an advantageous strategy to mitigate the characteristic beany taste associated with soy milk while maximizing nutritional benefits. The addition of flavourings and their respective quantities typically adhere to regulatory standards specific to each country (Jayalalitha et al., 2015).

The study of Jayalalitha (2015) focused on creating innovative, value-enriched yoghurt using a combination of soy milk and mango pulp. Initially, different levels of soy milk (0%, 10%, 20%, and 30%) were tested for inclusion in the yoghurt. Through optimization, it was determined that incorporating up to 30% soy milk enhances the yoghurt's protein content without compromising its physicochemical properties or sensory quality. Significant alterations in the protein and total solids content were observed between the control yoghurt and the value-enriched variant. The highest recorded values for protein and solid non-fat (SNF) content were 7.12% and 14.31%, respectively, in the yoghurt formulated with 30% soy milk and 15% mango pulp. Attempts were made to include dried mango pulp during culture inoculation. However, upon physicochemical and sensory evaluation, it was noted that yoghurt containing dried mango pulp could not be stored beyond 5 days at refrigeration temperature due to increased acidity, syneresis, unfavourable flavour, and reduced overall acceptability (Jayalalitha et al., 2015). The study's additional significant findings are presented in Table 2.

Table 2. Soy yoghurt with functional ingredients

Functional ingredient	Concentration of the functional ingredient	Proprieties	References
Red dragon fruit	25%, 30%, 25%	Antioxidant activity increased, while total LAB, pH decreased with red dragon fruit substitution. Viscosity decreased with the substitution of red dragon fruit because of its high water content Protein: 0.48%; Fat: 3.08% Carbohydrate: 3.46%; Moisture: 92.75%	(Marjan, 2023)
Moringa oleifera	0.1%	pH: 4.11; Ash content: 1.2% Protein content and fibre content increased The carbohydrate content decreased Incorporating Moringa root powder implies the potential for utilizing it as a dietary supplement to enhance the micronutrient content of food, thereby serving as a means for food enrichment.	(Ponka, 2022)
Scent leaf (*Ocimum gratissimum*) essential oil	0.5%	With the introduction of microcapsules containing essential oil derived from Scent leaf there was a notable result: the flavonoid content surged from 0.11 mg/100 g to 0.35 mg/100 g, while the FRAP content increased from 20.01 mg/100 g to 27.51 mg/100 g. Moreover, the iron chelating capacity rose from 7.50% to 11.08%, and the phenolic content escalated from 3.34mgGAE/g to 5.94mgGAE/g. Additionally, the DDPH value soared from 50.90% to 56.88%. Over five days at room temperature, there was a decrease in pH from 4.75 to 4.15, an increase in acidity from 0.71% to 0.99%, and a reduction in syneresis from 26.44% to 23.03%	(Olabiran, 2023)
Lemon grass	25, 50, 75 and 100 μL/L.	Moisture content range from 89.3% to 89.6% Protein content range from 5.5-6.8% Fat content range from 2.7 to 3.6% Total ash: 0.43-0.53%; Crude fibre: 0.06-0.33% pH range from 4.30 to 5.59 Titrable acidity: 0.01-0.07 g/L; Total phenolic compound: 8.59-18.40 mg/g	(Angelique, 2024)
Red fruit (*Pandanus conoideus* Lam.)	5%	pH: 3.91; Moisture: 38,97 % Total solids: 61.02%; Ash: 0.71% The results of the antioxidant activity evaluation for the red fruit combination yoghurt formulation revealed an IC50 value of 21.32 ppm, indicating a very strong antioxidant capacity.	(Tang'nga, 2019)
Mango pulp	5%, 10%, 15%	Fat: 3.11-3.41 %; Protein: 6.58-6.83% pH: 3.67-4.01; Acidity: 1.20-1.35% Soy milk incorporation increases the percentage of protein content with level of inclusion of yoghurt and mango pulp. Addition of fruit pulp also caused an increase in lactose content.	(Jayalalitha, 2015)
Lemon juice	1:9 2:8 3:7	Protein: 4.45-5.36% Fat content decreases with the juice addition from 1.74% to 1.64% Lactose content decreases with the juice addition from 1.35% to 0.67%; In fortified yoghurt- antioxidant content: 40.04 %	(Supriyanti, 2017)

Soy yoghurt with lemon juice

In accordance with the USDA National Nutrient Database, lemon (*Citrus limon*) typically contains approximately 53 mg of vitamin C per 100 grams, accounting for approximately 88% of the recommended daily intake. In this study, soy yoghurt fortified with vitamin C derived from lemon juice was produced. Previous research has not investigated the impact of adding lemon juice to soy yoghurt fortified with vitamin C on parameters such as protein, fat, lactose content, and the level of probiotics. The objective of Supriyanti et al. (2017) study was to examine the impact of incorporating lemon juice into soy yogurt. Various ratios of lemon juice were added to soy yogurt, in four different formulations: 0:10 (L0), 1:9 (L1), 2:8 (L2), and 3:7 (L3) followed by an analysis of their nutritional composition, including protein, fat, lactose, and probiotics. The resulting fortified yoghurt products were then evaluated to determine their respective levels of protein, fat, lactose, and probiotics. The study findings indicate that fortified yoghurt exhibited higher protein content compared to its original state. Moreover, among the probiotic yoghurt variants, L1 showed superior content compared to the L2 and the L3. From these observations, it can be inferred that L3 stands out as the most favorable option, boasting elevated protein levels, lower fat and lactose content compared to L1 and L2, while also containing probiotics

(Supriyanti, 2017). Table 2 presents more conclusive findings regarding the results.

4.3. Soy yoghurt enriched with aromatic plants

Aromatic plants used for enriching plant-based yogurt include *Moringa oleifera*, lemongrass, and *Ocimum gratissimum*, utilized as root powder, leaves, essential oils, and extract.

Soy yoghurt with Moringa Oleifera root powder

Utilizing *Moringa oleifera* root powder (MRP), rich in essential minerals and macronutrients, presents an innovative approach, when combined with soymilk, to enhance the nutritional profile of commonly consumed food items like yoghurt. However, it's crucial to meticulously remove the bark of moringa roots since it contains alkaloids, which can be toxic if ingested excessively (Alli et al., 2017).

The objective of the research of Ponka (2022) was to assess the sensory and physical characteristics of soy milk yoghurt fortified with moringa root powder. Incorporating soy milk enriched with Moringa notably elevates the fat, fibre, protein, copper, manganese and iron levels in the samples (Ponka et al., 2022). Other results are described in Table 2.

Soy-based yoghurt incorporated with scent leaf (Ocimum gratissimum) essential oil microcapsules

Scent leaf (*Ocimum gratissimum*) essential oil (OGEO) have been proven to possess antioxidants, antiseptics, antibacterial and antifungal activities (Olabiran et al., 2023).

The addition of OGEO microcapsules to soy-based yoghurt enhanced both its antioxidant and physicochemical characteristics, leading to an extended shelf life for the product. (Olabiran, 2023). The soy-based yoghurt containing *Ocimum gratissimum* essential oil (OGEO) microcapsules exhibited satisfactory physicochemical properties. Microcapsules encapsulating the essential oil extracted from *Ocimum gratissimum leaves*, along with cassava starch, gum arabic as cell wall material, and emulsifier, demonstrated potent antioxidant, free radical scavenging, and antibacterial properties when incorporated into soy-based yoghurt (Olabiran et al., 2023). Other important results are present in Table 2.

Lemon grass leaves extracts enriched soy yoghurt

Soybean-based food is a challenge in their utilization due to the undesirable associated flavours. The study of Angelique et al. (2024) focused on the preparation of soybean based yoghurt with different concentrations of lemon grass extract: 0, 25, 50, 75 and 100 μL/L.

The research findings indicate that adding lemon grass extract to soy yoghurt does not alter the basic nutritional composition of the yoghurt. However, it does elevate the content of soluble crude fibre and total phenolic compounds, while also leading to a decrease in pH. Furthermore, the inclusion of lemon grass extract enhances the sensory attributes of the yoghurt, including aroma, mouthfeel, and overall acceptance. Particularly noteworthy is the significant improvement in aroma and taste resulting from the addition of lemon grass extract to soy yoghurt, suggesting that the extract could potentially mitigate flavour-related issues commonly encountered in soy-based products (Angelique et al., 2024). The results more detailed are presented in Table 2.

CONCLUSIONS

The surge in interest for non-dairy plant-based yoghurts stems from various factors, including concerns for sustainability, health consciousness and dietary preferences, such as lactose intolerance and veganism. Scientific research emphasizes the significant positive impact of plant-based diets on ecosystems, biodiversity preservation, and climate change mitigation. Furthermore, studies analysing the health effects of plant-based diets indicate potential benefits, driving consumer demand for non-dairy alternatives.

Ingredients such as soy, oats, coconut, and innovative additions like tapioca, pineapple, strawberries, and lemon grass extract enhance the texture, flavour, and nutritional profile of plant-based yoghurts. Research on the production of non-dairy yoghurts highlights the importance of stabilizers, thickeners, and fortifying ingredients to achieve desired texture, stability, and nutritional content.

Innovations such as incorporating probiotics, fruit pulps, and functional ingredients like *Moringa oleifera* root powder and scent leaf essential oil microcapsules contribute to the development of diverse and nutritious non-dairy yoghurt options. While challenges exist, such as texture limitations compared to dairy yoghurts and the need for optimal fermentation conditions, ongoing research and innovation continue to address these issues, paving the way for further advancements in the field. In conclusion, the increasing popularity of non-dairy plant-based yoghurts reflects a broader shift towards sustainable, health-conscious food choices. With continued research and innovation, non-dairy yoghurts have the potential to meet the diverse dietary needs and preferences of consumers while contributing to a more sustainable and environmentally friendly food system.

As observed, there are still relatively few studies regarding the addition of functional ingredients in the production of soy yoghurts. However, there is a trend towards expanding consumer horizons to consume non-dairy products, to try new products that have functional properties that enhance a healthy lifestyle and help prevent the increasingly common effects of intolerances, with cases increasing from year to year. Transitioning to a more plant-based diet is crucial for promoting health and sustainability.

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RESEARCH ON EFFECT OF ACTIVE AND SUSTAINABLE ANTIMICROBIAL PACKAGING ON GROUND BEEF

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Abstract

Food packaging is innovating towards greener polymers and wider applications of bioactive compounds. Biodegradable active packaging based on natural compounds is a new approach, as it aims to improve shelf life. In this study, the active packaging materials used were chitosan/gelatin/clay polymer films in which sage, fennel, sea buckthorn nanoemulsions were incorporated. To carry out the experiments, the analysed meat was purchased from a chain of butchers in a store in Bucharest. For these experiments, chilled minced beef was chosen. Physical-chemical analyses were carried out on minced beef packed in chitosan/gelatin/clay polymeric films incorporated with nano-emulsions of sage, sea buckthorn, the control package being the polyethylene terephthalate casserole. The physical analyses performed during the experiments were: determination of free ammonia, water activity, colour analysis, analysis of dry matter and moisture content and determination of pH. The microbiological analyses consisted in determining the total plate count (NTC) and the presence of E. coli/coliform bacteria. Based on physical-chemical and microbiological analyses, both the freshness of the meat and its shelf life were determined, during seven days of storage (day 0, day 3, day 5 and day 7) at chilling temperature (4C). Following the results obtained in the physical-chemical analyses, we can state that there is a clear difference in the quality of the meat used in the experiments, during the storage period, depending on the packaging type used.

Key words: *food packaging, green polymer films, natural compounds, bioactive compounds, shelf life.*

INTRODUCTION

The accumulation of plastic waste in oceans and landfills has brought great threats to nature and human beings (MacLeod et al., 2021). Technological innovations in packaging are mainly related to the development of new polymeric materials and the combination of polymers with different properties (Espino-Pérez et al., 2016). It is necessary to develop sustainable packaging materials from biopolymers, which are renewable, degradable and compostable (Silva et al., 2020). Active packaging made of biodegradable polymers and natural additives is emerging as an ecological alternative to conventional packaging. Biodegradable active packaging based on natural compounds is a new approach, as it aims to improve shelf life and reduce food waste. Active packaging can be, for example, highlighted as having an antioxidant function capable of delaying or inhibiting the oxidation of packaged foods (Sharma et al., 2023).

Meat spoilage represents a great problem worldwide, affecting consumers health and leading to food waste, both having repercussions on economic aspects (Cercel et al., 2017; Popa et al., 2023). Research have been conducted in order to improve meat shelf life, minimize food waste and also minimise the impact that packaging have on the environment. Battisti et al. (2017) developed paper sheets coated with chitosan based solutions and packed beef meat. The packaging showed a lower microbial count in the packed beef samples compared to control, a greater stability in terms of lipid oxidation. New antibacterial packaging materials were developed by Lin et al. (2019), by incorporating chrysanthemum essential oil into chitosan nanofibers. The developed materials were tested on beef and showed an inhibition rate of *Listeria monocytogenes* of 99.91% at 4°C, 99.97% at 12°C and 99.95% at 25 °C, after 7 days of storage.

MATERIALS AND METHODS

Materials

To carry out the experiments, Figure 1, fresh minced beef was purchased from a chain of

butchers from a supermarket in Bucharest. Physical-chemical and microbiological analyses were carried out in triplicate during seven days of refrigerated storage at 4°C±0.5, namely on day 0 (initial analysis), day 3, day 5 and day 7 of storage. Therefore, for these experiments, 8 systems for minced beef packaging were prepared, as follows:
- Control sample, represented by commercial polyethylene terephthalate (PET) casserole - Control Sample;
- PET casserole + film of chitosan-gelatine-nano-clay-sage nanoemulsion - C/GE/Clay/Sage;
- PET casserole + chitosan-gelatine film- fennel nanoemulsion - C/GE/Clay/Fennel;
- PET casserole + chitosan-gelatine film- sage + sea buckthorn (SB) nanoemulsion - C/GE/Clay/Sage+SB;
- PET casserole + chitosan-gelatine film- fennel + sea buckthorn nanoemulsion - C/GE/Clay/Fennel+SB;
- PET casserole + Nanofiber electrospinning (ES) PLA - chitosan-gelatine-clay-sage nanoemulsion - ES PLA/C/GE/Clay/Sage;
- PET casserole + Nanofiber ES PLA- chitosan-gelatine-clay-sage nanoemulsion- ES PLA/C/GE/Clay/Fennel.

Figure 1. Aspect of packed beef using the developed packaging materials (own source)

Methods

During the refrigeration period, the following physical-chemical analyses were performed: determination of free ammonia (freshness analysis), water activity (a_w) index, colour analysis, moisture content and determination of pH. The microbiological analyses consisted of determining the total number of mesophilic aerobic germs and the presence of *E. coli*/ Coliform bacteria.

Briefly, free ammonia was determined using the Nessler reagent reaction method. Water activity index (a_w) of the tested samples was determined using a NOVASINA equipment by introducing the sample into specific recipients of the equipment and the value of a_w was read when stable at 25°C. Colour determination was performed at room temperature using a HunterLab colorimeter, Miniscan XE Plus. Moisture content was performed by weighing 5 g of sample, which was further subjected to drying at 105°C using a RADWAG MAC 50 thermobalance. The results were expressed as a percentage (%). The pH determination was performed using a pH meter WTW INOLAB 720 series type with automatic temperature compensator. The microbiological analysis aimed at determining the total number of aerobic count and the presence of *E. coli*/Coliforms in the studied samples. For these analysis, specific Petri films with lyophilised culture media were used.

RESULTS AND DISCUSSIONS

Free ammonia

According to the results of the freshness analysis, the samples presented a negative Nessler reaction for 5 days, except the Control sample which presented a positive reaction. After 7 days of analysis, all samples presented positive Nessler reaction, meaning that their freshness started to degrade.

Water activity

Water has an important role to ensure food quality; its presence in food in certain amount provides both conditions for the development of spoilage microorganisms and enzymatic reactions that are directly responsible for the smell, colour and taste of food. The values of the water activity index (a_w) of the analysed samples during storage, are presented in Figure 2.

Water activity presented higher values for the Control sample with an increasing tendency over the storage period. For all samples packed in the developed packaging systems there can be observed that the values of a_w presented values lower compared to the Control sample, with a tendency of decreasing over the storage period. The lowest values were determined for samples ES PLA/C/GE/Clay/Sage and ES

PLA/C/GE/Clay/Fennel. This leads to the conclusion that the water available for the development of microorganisms has decreased, thus preventing the alteration of the product from a microbiological point of view.

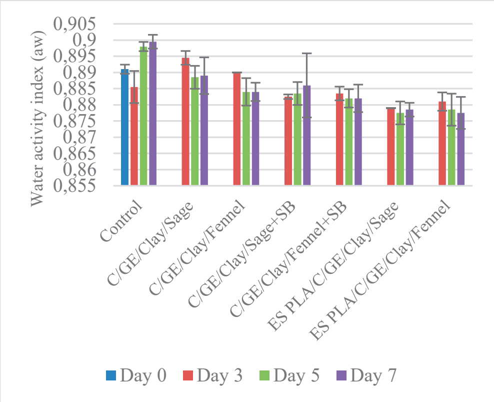

Figure 2. Water activity index (aw) values determined during the storage period at 4°C

Moisture content

The moisture content for the analysed samples is presented in Figure 3. On the last day of analysis, the Control sample registered a decreasing value of this parameter compared to the initial moment of analysis and compared to all analysed samples. During the storage period small variation were registered for the samples packed using the developed materials, however the obtained values were similar to the first day of analysis (Day 0).

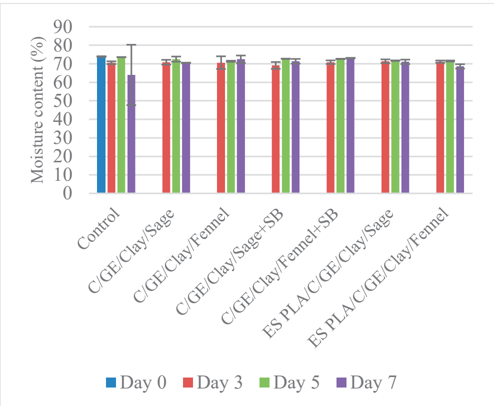

Figure 3. Values regarding the moisture content of minced beef during the storage period at 4°C

Determination of pH

The pH value indicates a number of characteristics of minced beef, such as colour, freshness, water holding capacity or juiciness. According to the results obtained (Figure 4), the meat samples packed in the active films and stored at a temperature of 4°C maintained until the seventh day pH values lower than 6, while the meat packed in PET casserole reached a pH of 6.21. For all samples these pH values are normal for beef stored at refrigeration temperature. It seems that the Control sample has an increased aging rate than samples packed in active films.

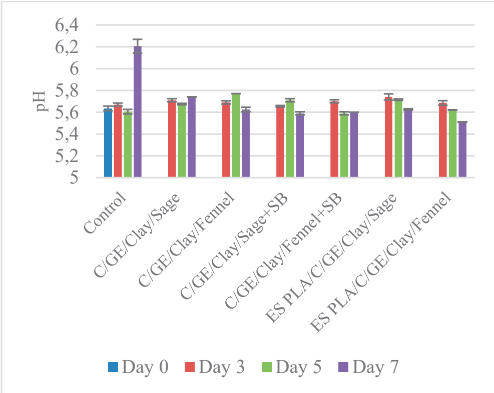

Figure 4. The evolution of the pH values of minced beef, during the storage period at the temperature of 4°C

Colour analysis

In the food industry, the colour of the products is an important attribute, because with its help the quality is appreciated. Colour measuring instruments are used to control the colour of ingredients and to evaluate the efficiency of processes in obtaining and maintaining the desired coloured food product. Colour measurements are also made for other purposes. These include: measuring raw and processed food quality indices for the use of data in quality control, documentation and communication; determining the conformity of food quality according to specifications; analysis of changes in the quality of food products as a result of food processing, storage and other factors. The results obtained following colour analysis are presented in Tables 1-3.

Table 1. Evolution of the L* parameter values for the studied samples

Sample / Moment of analysis	Day 0	Day 3	Day 5	Day 7
Control	33.079 ± 0.77	35.198 ± 0.37	31.119 ± 0.18	30.516 ± 0.21
C/GE/Clay/Sage	33.079 ± 0.77	30.610 ± 0.56	33.404 ± 0.27	30.815 ± 0.25
C/GE/Clay/Fennel	33.079 ± 0.77	29.160 ± 0.34	30.695 ± 0.12	33.138 ± 0.57
C/GE/Clay/Sage+SB	33.079 ± 0.77	29.817 ± 0.09	33.876 ± 0.36	32.599 ± 0.71
C/GE/Clay/Fennel+SB	33.079 ± 0.77	32.319 ± 0.31	29.453 ± 0.25	29.469 ± 0.32
ES PLA/C/GE/Clay/Sage	33.079 ± 0.77	31.302 ± 0.46	30.952 ± 0.38	41.423 ± 0.34
ES PLA/C/GE/Clay/Fennel	33.079 ± 0.77	30.457 ± 0.21	32.555 ± 0.16	30.701 ± 0.45

Table 2. Evolution of the a* parameter values for the studied samples

Sample / Moment of analysis	Day 0	Day 3	Day 5	Day 7
Control	17.123 ± 0.63	8.788 ± 0.18	11.597 ± 0,91	14.431 ± 0.34
C/GE/Clay/Sage	17.123 ± 0.63	9.657 ± 0.20	10.607 ± 0,31	12.821 ± 0.71
C/GE/Clay/Fennel	17.123 ± 0.63	11.715 ± 0.06	10.141 ± 0,58	7.643 ± 0.10
C/GE/Clay/Sage+SB	17.123 ± 0.63	10.193 ± 0.25	14.122 ± 0,20	9.620 ± 0.42
C/GE/Clay/Fennel+SB	17.123 ± 0,63	9.321 ± 0.20	11.986 ± 0,20	13.119 ± 0.51
ES PLA/C/GE/Clay/Sage	17.123 ± 0.63	10.950 ± 0.19	8.916 ± 0,64	7.257 ± 0.15
ES PLA/C/GE/Clay/Fennel	17.123 ± 0.63	19.550 ± 0.57	9.720 ± 0,42	12.319 ± 0.50

Table 3. Evolution of the b* parameter values for the studied samples

Sample / Moment of analysis	Day 0	Day 3	Day 5	Day 7
Control	16.750 ± 0.45	13.521 ± 0.10	14.257 ± 0.14	15.453 ± 0.13
C/GE/Clay/Sage	16.750 ± 0.45	13.440 ± 0.11	13.959 ± 0.14	13.920 ± 0.34
C/GE/Clay/Fennel	16.750 ± 0.45	14.235 ± 0.26	14.696 ± 0.17	12.925 ± 0.32
C/GE/Clay/Sage+SB	16.750 ± 0.45	14.102 ± 0.27	15.996 ± 0.20	14.062 ± 0.19
C/GE/Clay/Fennel+SB	16.750 ± 0.45	13.703 ± 0.13	14.307 ± 0.19	13.497 ± 0.20
ES PLA/C/GE/Clay/Sage	16.750 ± 0.45	14.088 ± 0.14	13.421 ± 0.15	15.155 ± 0.14
ES PLA/C/GE/Clay/Fennel	16.750 ± 0.45	16.785 ± 0.34	13.702 ± 0.19	13.703 ± 0.14

In general, the values of the parameters L*, a* and b* were slightly lower during the analysis period compared to the values obtained initially (Day 0). Therefore, the luminance of the samples (L*) showed lower values on days 3, 5 and 7 of the analysis, which shows that the samples had a slightly darker colour compared to the initial time of analysis. This is normal under the conditions of keeping the meat refrigerated, due to curing process. The values of the parameter a* decreased significantly compared to the initial moment of analysis for all the samples studied, a fact that shows the shift to a darker colour of the samples. A decrease in the values of this parameter was also observed in other studies and may be due to the oxidation of oxymyoglobin and metmyoglobin (Suo et al., 2016). The same trend was observed in the parameter b*, with lower values during the analysis period compared to the initial moment. Similar results were observed by Pereira Cardoso et al. (2016), who studied the effect of gelatine and chitosan-based coatings on beef colour. In this study, lower values of the L* parameter were also observed compared to the initial moment of analysis, this may be due to the coatings used.

Microbiological analyses

Microbiological analyses were performed to highlight the development of aerobic mesophilic bacteria, coliform bacteria and the presence of E. coli for the samples used in the experiments (Table 4). The results showed that in the samples that are stored in polymer films containing oils, the number of mesophilic aerobic germs is much lower, compared to the Control sample, which is stored in PET packaging. The ground beef subjected to the experiments was not contaminated with E. coli. In none of the 7 days of analysis were found E. coli colonies, neither in the Control sample nor in the samples packed in active films. After the first 24 hours of storage, colonies of coliform bacteria were found in the minced beef from the Control sample, however coliforms were further determined only in the Control sample during storage, while the active packaging proved to be efficient in the inactivation of this type of bacteria during storage.

Table 4. Microbiological analysis results - total number of aerobic mesophilic germs in ground beef samples stored at 4ºC

Analysed parameter / Storage time (days)	Total Plate Count (log CFU)				*E. coli* / Coliforms (log CFU)			
	0	3	5	7	0	3	5	7
Control	2.92	3.72	4.36	5.4	- / 2.04	- / 3.36	- / 3.61	- / 3.98
C/GE/Clay/Sage	2.92	3.04	3.93	4.98	- / 2.04	- / -	- / -	- / -
C/GE/Clay/Fennel	2.92	3.56	2.08	4.14	- / 2.04	- / -	- / -	- / -
C/GE/Clay/Sage+SB	2.92	3.27	4.11	4.34	- / 2.04	- / -	- / -	- / -
C/GE/Clay/Fennel+SB	2.92	3.72	4.12	5.32	- / 2.04	- / -	- / -	- / -
ES PLA/C/GE/Clay/Sage	2.92	3.84	4.29	5.07	- / 2.04	- / -	- / -	- / -
ES PLA/C/GE/Clay/Fennel	2.92	3.41	4.08	4.61	- / 2.04	- / -	- / -	- / -

- absence of microorganism

CONCLUSIONS

According to the obtained results, the minced beef samples packed in the presence of the six studied films (C/GE/Clay/Sage; C/GE/Clay/Fennel; C/GE/Clay/Sage+SB; C/GE/Clay/Fennel+SB; ES PLA/C/GE/Clay/Sage; ES PLA/C/GE/Clay/Fennel) and stored at 4°C demonstrated a good behaviour for 5 days, while the Control sample started the process of degradation after only 3 days from packaging. The microbiological analyses showed that the microbial load of the tested samples had a continuous decreas during the refrigeration period for all the analysed samples compared to the control sample. Therefore, the developed packaging materials could be used for meat packaging, showing good properties in terms of shelf life prolongation.

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FEASTING ON FUNGI: EXPLORING FUTURE PERSPECTIVES OF CONSUMERS PREFERENCES AND COMMERCIAL PRACTICES ON THE GROWING MARKET OF PLANT-BASED FOODS

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Abstract

Consumer's dietary preferences are an ever-evolving mechanism subject to a series of dynamic factors, including their growing consciousness towards healthier food alternatives and sustainability. As industry's focus tends to extend beyond conventional plant-based options, innovative results shed light on something deep beneath the surface, rooted in mycology. This study aims to investigate the intricate factors influencing the preferences and commercial behaviour of the Romanian consumer and how likely they are to accept and integrate Mycelium-based products into their current diet. The research involved collecting data through a questionnaire, which was subsequently completed by a target group, forming the representative sample. By interpreting the results obtained from data processing and statistical evaluation, we gained access to valuable insights regarding both the burgeoning field of alternative protein sources and how they link to the broader context of emerging trends in consumer behaviour.

Key words: *behaviour, healthier food, mycelium based, plant-based, protein.*

INTRODUCTION

The modern man is facing a handful of dilemmas when it comes to his dietary choices. It is a well-known fact that agriculture, that is to say, conventional protein-producing systems, entails vast areas of land that are left unavailable to natural ecosystems. The influence of Anthropogenic factors is pivotal to the increasing statistical data regarding water pollution, greenhouse gas emissions, and land depreciation. Over the past decades, it has become increasingly clear that large-scale agriculture operations come with the heavy cost of durable development and prove to be less and less viable. The multifaceted nature of the present issues extends beyond the business strategy of big agricultural conglomerates, presenting itself as a shared challenge that confronts the health of our global community. Dietary risk factors, such as insufficient intake of vegetable fibre coupled with elevated intakes of processed red meat, play a significant role in the prevalence of chronic non-transmittable diseases, especially type II diabetes and coronary heart disease and some cancers (Pan, 2012; Popkin, 1994; Micha, 2010; Key, 1999; Fung, 2004).

The Rockefeller Foundation-Lancet Commission on Planetary Health suggested that there is major potential for dietary changes to improve health and reduce the environmental impacts of food production, while the United Nations Food and Agriculture Organization (FAO) defines sustainable diets as those that are healthy, have a low environmental impact, are affordable, and are culturally acceptable (Burlingame & Dernini, 2010; Whitmee et al., 2015).

Interplays of psychological factors influencing taste preference, sociological factors, and the economic component dictating accessibility and affordability are the main drives behind consumers' choices and ultimately decide what sort of products will stand the test of time on the market. The emergence of various individual lifestyles and trends promoted in both urban and rural communities have generated in the last decades a plethora of eating regimes (Poșan et al., 2022), with flexitarian (a fusion between "flexible" and "vegetarian", referring to an individual who follows a primarily but not strictly vegetarian diet) gaining significant traction. Nonetheless, protein intake is important in achieving a well-functioning and balanced organism because it facilitates the development and repairing of muscles and tissues and the transportation of nutrients (Barbu, 2022; Popa, 2019).

In response to aforementioned conditions and consumers expectations, the food industry has started placing a greater emphasis on developing products that feature vegetable protein as a traditional meat analogue. It is estimated that the plant-based food market will further grow from approximately $US 30 billion in 2020 to $US 160 billion by 2030. (https://www.foodmarket.com).

Although the plant-based market has seen a stagnant year for meat substitutes in the United States, the European customer is only beginning to familiarise with alternative protein sources thus projecting an upward trajectory of sales and increase in demand.

Unlike soy, peas or wheat protein, mycelium-based products have long fibrous filaments that mimic muscle structures, an important sensory characteristic that can ease the preparation process and by so attract a broader range of customers. Additionally, they present a highly nutritious profile consisting of vitamin B12, fibre and complete protein that varies up to 15g per 120 g of classic steak, lacking only in saturated fats (Watson, 2023)

The paper's main objective is to examine critical aspects of fungal protein that play an essential role in determining consumer acceptance of the end product. The primary focus is on the marketing aspects and the collective image associated with terminologies such as plant-based protein, alternative meat, and fungal proteins. The study is centred on the Romanian retail market. While Romanian consumers have historically integrated mushrooms into their diets, our cultural and empirical observations suggest a lingering attachment to traditional meat sources, particularly in rural communities where meat remains a component of subsistence agriculture. However, owing to the globalisation of the food market, there is a growing demand for innovative and eco-friendly products. Our purpose is to explore the factors that specifically influence the acceptance and purchase of plant-based products, emphasising the look and feel of traditional benchmark items such as steaks, burger patties, sausage, and bacon.

This study aims to investigate the intricate factors influencing the preferences and comercial behaviour of the Romanian consumer and how likely they are to accept and integrate Mycelium-based products into their current diet.

MATERIALS AND METHODS

Materials

The study conducted by us involved 138 individuals interviewed; of these, 89 were female and 48 were male (one person chose the response option "I prefer not to specify"), with representation from both urban (105 individuals) and rural community (33 individuals). To determine if the objectives pursued in the study vary by age, one of the items targeted this characteristic. The participants in this study fall into the following age categories: 18-21 years old (43.5%), 22-30 years old (30.4%), 31-40 years old (5.1%), 41-50 years old (7.2%), and over 50 years old (13.8%). Additionally, the level of education represents an influential factor in assessing our consumers' profiles. For this item, we had six response options: ongoing/completed high school studies (2.9%), ongoing/completed vocational studies (2.9%), and ongoing university studies (60.2%). completed university studies (15.2%) ongoing or completed postgraduate studies (18.8%).

Methods

The research instrument used is represented by the questionnaire. Sixteen items were developed, grouped into 2 sections: socio-demographic data (5 items) and consumer profile (11 items).

Data processing methodology

The data were processed according to descriptive statistical methodology, and the results are presented using adequate techniques. It is important to note that the variants derived from a questionnaire inherently reflect a categorical spectrum, illustrating the diverse intensity of responses. When respondents categorically express their interest, it indicates a clear and unequivocal inclination toward the subject matter. As the likelihood of interest increases, the nuances become more apparent, suggesting a high degree of engagement. Moving along this continuum, the acknowledgment that there is a possibility of interest signifies a potential but not definitive involvement. Conversely, when respondents answer that they wouldn't be interested, it represents a decisive and unambiguous disinterest in the subject matter.

RESULTS AND DISCUSSIONS

As expected, the major force driving consumer's decision to try out a new food product is taste with a score of 54.34%, followed closely by personal experience and familiarity with the respective product at 44.2%. In terms of external influences, respondents answered in proportion of 70.29% that friends 'sometimes' affect their decision to try out a new product, confirming former studies that suggest people are more likely to adapt their eating behaviour similarly to their peers (Figure 1).

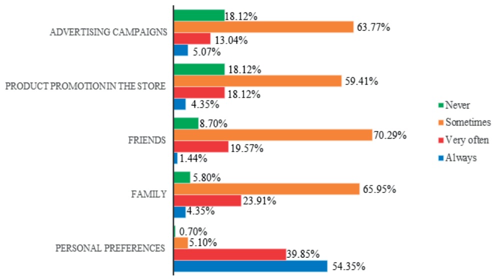

Figure 1. The frequency of factors influencing the testing of a food product

Notably, despite the fact that product promotion in stores and advertising campaigns conventionally have a significant impact on consumers choice, our respondents have shown a significant disinterest in both, scoring only a flat 18.12% (Boateng, 2015). This aspect can be explained by the fact that consumers consider their own experience with the product more than advertising campaigns. When asked if they were willing to try out this type of product if it would receive public endorsement from a trusted source or public person, almost half of our respondents (49.6%) said the likelihood is very high depending on the source and 24% categorically agreed. This information is in concordance and reflects the cultural shift that social media has generated and the outsourcing of varying information consumers are being exposed to. Public figures and institutions can influence food choices through policies, health campaigns or just general advocacy for specific products, diets and lifestyles (Kamboj & Sharma, 2022; Vithayathil et al., 2020). Bearing in mind the financial considerations and how price can represent a substantial barrier in purchasing food, the need for a balanced price-quality ratio is a calibrating factor in the psyche of the Romanian consumer. 44.93% of our respondents said they 'always' take this criterion into account. Food preferences are strongly influenced by product characteristics such as labeling and packaging (Loose, 2012) The manufacturer (Bahaciu, 2019), price (Barbu, 2023) discounts and package design (Loose & Szolnoki, 2012) equally accounted for 31.88% (Figure 2).

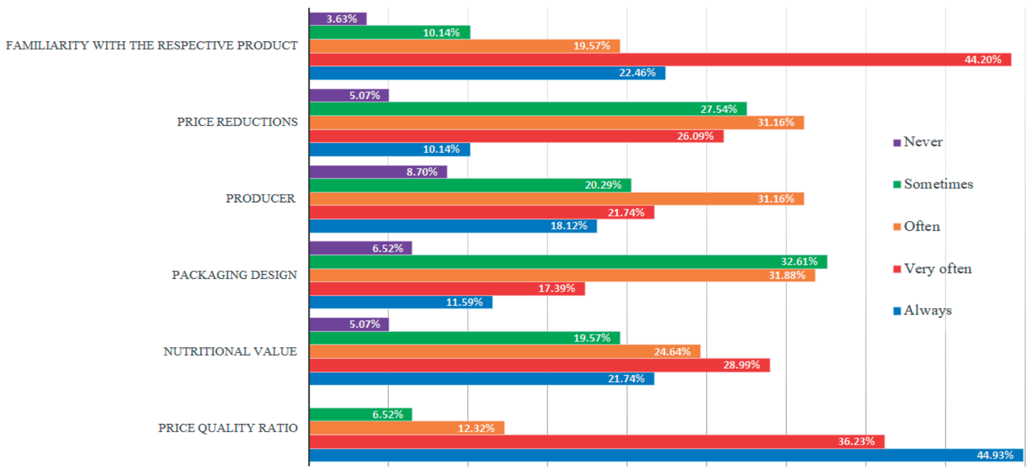

Figure 2. The frequency of factors influencing the acquisition decision

Consumers have different opinions regarding testing or consuming innovative products. Some of them may have reservations regarding the food safety of said products or may be resistant to change, preferring products that are more familiar. Another category is interested in new culinary experiences. Additionally, many consumers appreciate innovative food products because obtaining them has a low environmental impact. The majority of respondents (40.9%) stated that they are aware, and concerned about the environmental impact. Approximately one-third of the study participants mentioned: "Yes, I am aware, but I am not concerned about this aspect". Surprisingly, one-fifth of respondents specified that they do not have a clear opinion on this matter. Only 5.1% of study participants are not aware or interested in this issue (Figure 3).

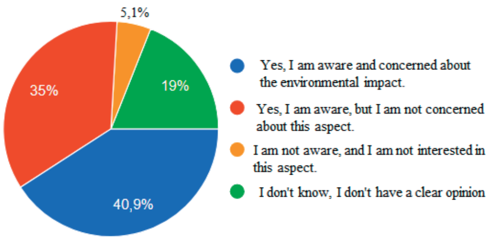

Figure 3. The level of awareness of the environmental impact

More recently, individuals have found themselves in the situation where their schedules are increasingly dominated by their professional commitments.

Frequently, preparing lunch and dinner at home is reserved for the weekends, while on weekdays the prevailing practice is to order food via food service delivery Currently, the restaurant industry needs to adapt to consumers' preferences for competitive pricing, convenience, and reliable food delivery (Teo et al., 2024). Following our results, the response options integrating alternatives for situations when a meal cannot be prepared in house were:

a. Restaurant/bar/café: 17.39% of respondents specified that they do it "always". Those who "never" choose to eat at a restaurant are in a relatively small proportion (9.42%). The majority of respondents mentioned that they "sometimes' opt for this choice (Figure 4).

b. Food delivery services: - Approximately half of the interviewees (45.65%) use this type of service only "sometimes". About one-third of the study participants "usually" use these services. Those who "never" or "always" use food delivery services are approximately equally distributed.

c. Snacks that can provide me with an optimal number of calories - For this variant, opinions were somewhat evenly split between those who "usually" or "sometimes" take into account having an efficient calorie intake through snacks, and those who mentioned that they "always" ensure their calorie intake is balanced out through snack were twice as many compared to those who specified that they "never" do this.

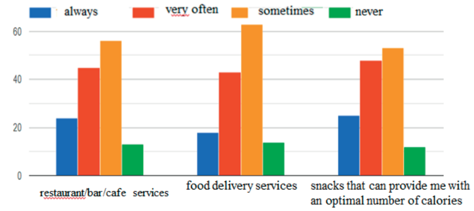

Figure 4. Options for meal service

In order to make a clearer analysis of consumer acceptability of replacing animal products with plant products, we investigated how often consumers eat vegetables or products made using only plant-based ingredients, and the results showed the following: 46.38% of respondents eat vegetables daily, and 34.06% eat products derived from plant-based ingredients several times a month. Also, 52.17% of the respondents ticked "several times a month" when asked how often they eat mushrooms, which indicates that our innovative product could incite our consumers' interest (Figure 5).

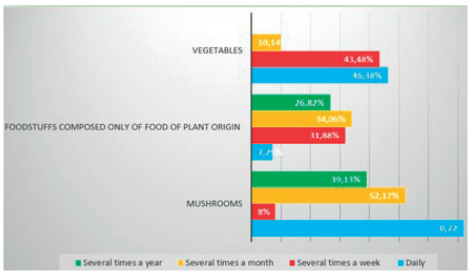

Figure 5. Frequency of consumption for plant-based foods

When asked "why would you replace animal dishes with similar vegetable dishes", consumers answered "very often" in the case of them being tastier (32%) and "sometimes" if the products looked more appetising (35%). Based on this, we can observe that sensory stimuli are an important factor to consider when creating a new product (Figure 6). Abdelaziz (2022) conducted a study in 2000 aiming to ascertain whether attitudes could be formed involuntarily during the categorization of a new hybrid product under the influence of associated sensory stimuli (smell and taste). The study's results indicated that study participants were able to form both implicit and explicit attitudes towards the tested product after exposure to sensory stimuli.

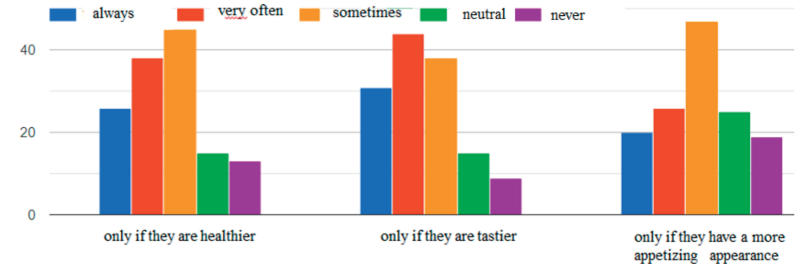

Figure 6. The reason for replacing animal-based dishes

For item 15 our respondents were shown an image depicting a clearly tender, juicy piece of steak and were asked how does the image affect their appetite. The appetite is deeply connected to the sensory experience - in our case cutting through the steak and witnessing its texture and flavour. We took into consideration the data resulting from item 14 which reflected that for our consumers the top characteristics of products like steak, burger patties and bacon are most frequently taste, smell and texture (Figure 7).

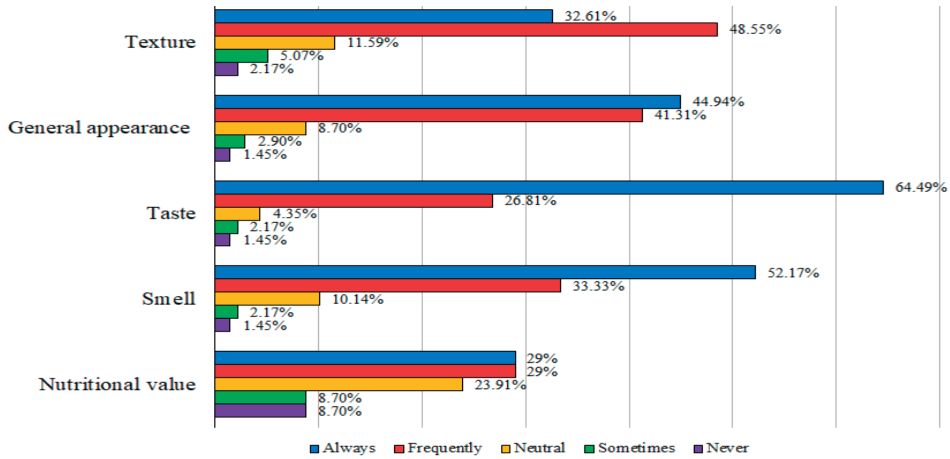

Figure 7. Specific characteristics of steak, meatball, or bacon-type products

This combination of characteristics is more likely to heighten the sense of appetite among consumers and is associated, according to almost 50% (67 respondents), with a savoury meal.

Item 16 represents the final questions that concludes our survey and a conceptual exercise we asked our respondents to complete: how would their perception change if they were to find out that the product shown previously is entirely made out of mycelium. Subsequently, respondents are prompted to reflect on how this unexpected revelation influenced their perception of the product. Almost 38% of them

(52 respondents) would categorically be interested to try it out, while 55 respondents claimed it is very likely they would be interested in cooking it themselves. A similarly high percentage of respondents would rather purchase it in store, already cooked. These results collectively suggest that there is a substantial segment of the population willing to embrace and incorporate innovative plant-based products into their diets, with preferences ranging from hands-on cooking experiences to the convenience of pre-prepared options available in stores (Figure 8). These insights can be valuable in assessing food consumption patterns and ultimately for product development. It is important to keep in mind that other factors such as proper labelling and marketing positioning will have a great impact on the acceptance of this new-wave of products (Constantin et al., 2009). Safety regulations that mandate clear and accurate labelling not only empower consumers to make informed choices but also foster a sense of security and confidence in the products they purchase.

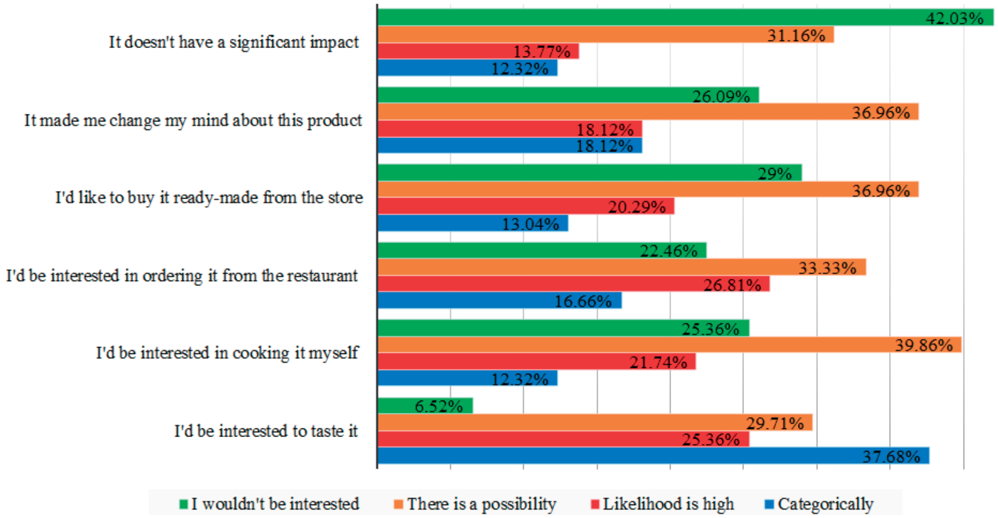

Figure 8. Consumer perception

CONCLUSIONS

The principal objective of our study was to assess the attitude and degree of acceptance exhibited by Romanian consumers toward mycelium-based products - an innovative category within the spectrum of sustainable food alternatives. The analytical exploration via a questionnaire of 16 items encompassed diverse dimensions of consumer behaviour and preferences, yielding insights into the prospective trajectory of mycelium-based products within the Romanian market.

Over 50% of our respondents confirmed they consume mushrooms monthly, suggesting a potential openness towards products derived from fungal protein. A noteworthy segment of our respondents displayed concern about the environmental repercussions of their food choices, revealing an emergent trend towards eco-conscious consumerism. This trend converges with the escalating popularity of plant-based alternatives, underscored by dual considerations of environmental sustainability and a proclivity for novel sensorial experiences. However, the primacy of taste and overall sensory experience prove to be the main agents in shaping consumers choices. This particular aspect might suggest an openness to trying novel tastes and textures as long as the product maintains its savoury and flavourful profile and it is as easy and convenient to integrate into daily consumption habits as a home-delivered burger. The sustained emphasis on a balanced price-quality ratio many respondents opted for stays consistent with previous findings wherein financial considerations occupy a central position in the scheme of the consumer decision-making tree. This underscores a pivotal tenet for brands operating with mycelium-based products: the imperative to provide consumers with a compelling value proposition that delivers both affordability and product quality.

This dual consideration introduces a strategic dilemma for developers and market strategists. On one hand, there is an avenue to position these products as a more economical alternative to conventional meat, thereby catering to a broader consumer base. This approach involves a deliberate effort to offer price points that are not only competitive but also significantly lower than traditional meat options. By doing so, developers can effectively tap into the market segment characterised by cost-conscious consumers, thereby expanding the reach and accessibility of mycelium-based products.

On the other hand, there lies an opportunity to position mycelium-based products in the high-end segment, vouching for superlative quality. In this scenario, developers can leverage the unique qualities of mycelium-based products - whether it be their sustainability, nutritional profile, or distinct sensory attributes - to justify a premium price point. This strategic positioning could target consumers who are willing to invest in products that align with their preferences and values. The impact of social networks on consumer decisions, coupled with a palpable receptiveness to products endorsed by trusted entities, delineates a degree of reliance on social circles and influential public persons, consolidating once again that identity validation exists within our food choices. These insights imply that strategic endorsements and social validation could play a pivotal role in fostering acceptance for mycelium-based products, aligning with broader societal trends.

In the end, while our study provides valuable insights into the acceptance of mycelium-based products among Romanian consumers, it is essential to acknowledge certain limitations. The sample size and demographic representation may not capture the full spectrum of consumer diversity, and the reliance on survey responses introduces potential biases. Additionally, the dynamic nature of consumer preferences poses challenges in offering a definitive and timeless analysis. Nevertheless, we perceive these limitations not as constraints but as opportunities for further research refinement. The information uncovered in this study creates a groundwork for more nuanced investigations. Despite these considerations, we remain cautiously optimistic about the prospect of integrating mycelium-based products on the Romanian market and into our dietary choices. We believe the modern man might find the solution to his handful of dilemmas through an integrative approach rather than opting strictly for plant-based or traditional steak.

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ADVANCES IN BIO-BASED FOOD PACKAGING MATERIALS - A REVIEW

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Abstract

In the recent years there has been a constant need for the development of novel packaging materials, which can be defined as made from materials derived from renewable sources, that can provide alternative and sustainable routes for the food packaging industry in order to replace the petroleum-based polymers with bio-based materials. A constant concern for the environment wellbeing has been the agricultural waste which is not valorised in a sustainable matter but rather incinerated or disposed of in landfills. A novel way to promote a circular bio economy would be to utilise these agri-food waste and by-products for the development of novel and sustainable packaging materials. This article aims to review the development of bio-based packaging materials and production technologies considering by-products and waste minimization, recyclability, biodegradability, and their impact on the circular bio economy and sustainability.

***Key words**: agricultural waste, circular bioeconomy, sustainable packaging materials.*

INTRODUCTION

One of the main problems that the industry in general, and the food packaging industry in particular, will have to solve in the next few years is that of the resources it uses. Currently, the raw materials used to meet most of the needs of human society are based on fossil fuels (Yi et al., 2023). Most of the researchers believe that the reduction of the fossil resources stock is inevitable and even their exhaustion in the course of a few decades (Azni et al., 2023).
Plastic materials have become more and more important during the last century, finding applications in the most diverse fields of industry and the life of the common human beings. Plastic materials are a convenient solution for many technical fields, due to their good physical-mechanical characteristics, their low weight and reasonable price. The main disadvantage is given by the long-term pollution effect they produce after their lifespan (Gustavsson et al., 2017; Solarin, 2020). In recent years, in the food packaging polymer industry, there is a tendency to replace conventional plastic materials with ones that show improved biodegradability or, even better, with ones that come from renewable resources and are susceptible to biodegradation (Rai et al., 2021). The main issue for the food packaging industry is the elimination of harmful substances that could be in the packaging materials, recyclability, safety and easy separation of materials in order to be recycled, use of recycled resources and handling precautions (Mangaraj et al., 2019; Tajeddin & Arabkhedri, 2020; Motelica et al., 2020).
The newest economic interest is the use of resources of renewable plastic materials as a respect for the environment, and as an ecological management for exhaustible fossil materials. From this perspective, plant raw materials and biopolymers of this origin possess properties of great interest in the plastics industry such as: biodegradability, biocompatibility, selective permeability and modifiable physical-mechanical properties (Asgher et al., 2020).

Bioremediation - an emerging approach towards environment restoration

Environmental bioremediation represents a new field within biotechnologies. Bioremediation is a method of eliminating pollutants from the environment with the help of microorganisms, by transforming them from toxic compounds into non-toxic compounds, without affecting the environment (Mehrotra et al., 2021). In other words, bioremediation is a technology that aims to remove pollutants from the environment, restoring the original natural environment and preventing future contamination (Gu, 2021). These microorganisms can be isolated from contaminated areas or from other sources and transferred to polluted regions. Starting from the degradative activities of microorganisms, numerous depollution technologies have been researched and developed, which are included generically in the term bioremediation (Pande et al., 2020). The control and optimization of bioremediation processes is a complex system that involves several factors including: the existence of a microbial population with a high capacity to degrade polluting compounds; the availability of some contaminants in the populations of microorganisms; environmental factors (soil type, temperature, pH, presence of oxygen and other acceptors, nutrients) (Ayilara & Babalola, 2023; Kapur et al., 2023). Microorganisms can be isolated from almost all environmental conditions such as from regions with low temperature below zero degrees, from regions with very high temperatures, desert conditions, from water, etc. (Hlihor & Cozma, 2023). The most important factor for these microorganisms to survive is the carbon source and the energy source.

MATERIALS AND METHODS

The information and data presented in the review article is composed of novel researches made in the past decade regarding the advances in bio-based food packaging materials. The analysis focused in the importance of developing bio-based materials for the food packaging industry in order to replace the fossil fuel ones, the methods of bioremediation and biodegradation and the impact on the environment. Another critical point of the research was to gather the latest applications of the bio-based packaging materials and give a clear example on what food products were they used on. Web of Science, Elsevier, PubMed, ScienceDirect, JSTOR and Springer databases were electronically searched for articles published in the last decade. The literature search included document types such as research articles and reviews, on the following topics: „bio-based materials", „bioremedation", „food packaging sustainability", "food packaging biopolymers". Research articles published in the last decade were gathered and synthetized from publisher databases in order to give an in-depth view of the discussed subject.

RESULTS AND DISCUSSIONS

The biodegradability of polymers is influenced by numerous structural parameters and by the use of statistical data interpretation methods which. Through these methods it can be established which of them correlates best with biodegradability (Idrees et al., 2020; Van der Zee, 2020). Most natural polymers such as starch, cellulose and proteins are easily biodegraded by hydrolysis followed by oxidation with the help of enzymes (Bahl et al., 2021). In order to evaluate the biodegradation process, it must be taken into account that the molecular properties of the polymer, such as the distribution of molecular masses, crystallinity and morphology, will dictate the physical properties of the packaging products obtained from these polymers (Glaser, 2019; Mangaraj et al., 2019).

Characteristics and types of biodegradable plastics

In the recent decades, plastic has conquered the world and penetrated all spheres of our life from industry to everyday life: plastic windows, tableware, furniture, packaging, almost everything we use is made of plastic or contains the material plastic. The production of biodegradable plastics and food packaging reached its peak in 2010, when several major companies producing bioplastics manufactured products from a large set of raw materials (Haider et al., 2018). In the last decades, synthetic polymers are used on a large scale in many fields of activity. These macromolecular substances are usually of petroleum-based origin and are not biodegradable (Gowthaman et al., 2021). However, oil resources are limited and the use of non-biodegradable polymers causes serious environmental problems.

Many solutions have been proposed for waste management such as recycling, incineration and degradation of food packaging materials. Quality products are not obtained through recycling due to the heterogeneous nature of plastic materials and the incineration of plastic materials with the release of toxic gases and vapours can be a serious health hazard, thus the most appropriate solution is represented by using biodegradable food packaging materials (Shaikh et al., 2021; Panou & Karabagias, 2023).

Technologies for obtaining new polymer materials are very promising from the point of view of applications, such as: agriculture (films, products with herbicidal action), consumer goods (packaging) with a determined life span, products based on polymer matrices with controlled release of active substance (medicines), recovery and recycling of polymer waste, medicine and implants (To'ychiyev & Soliyev, 2022; García-Collado et al., 2022). Current trends in the science of polymer processing are oriented towards the creation of new types of materials with biodegradability properties (Figure 1), biocompatibility, corrosion resistance, flexibility, optical and electrical properties, to replace the materials traditionally used in agriculture, electronics, industry, medicine and the possibility of recovery and recycling of these materials to protect the ecosystem (Terzopoulou et al., 2022; Gnanasekar et al., 2023).

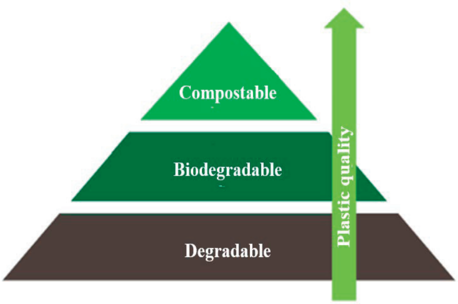

Figure 1. Novel types of biodegradable materials

On a worldwide level, the idea is accepted that the prolonged use of polymers for applications that require a short lifetime (packaging, food industry, surgery, hygiene) is not entirely adequate. This is not recommended when there are concerns about preserving ecological systems (Nanda et al., 2022). Most synthetic polymers are obtained from petroleum resources and are not biodegradable. Their introduction in the manufacture of landmarks for industrial purposes is not negligible. Obtaining biodegradable polymer mixtures constitutes a priority and multidisciplinary research direction, closely connected with fundamental research in the field of thermodynamics and polymer compatibility, environmental engineering, biotechnologies (Rajeshkumar, 2022).

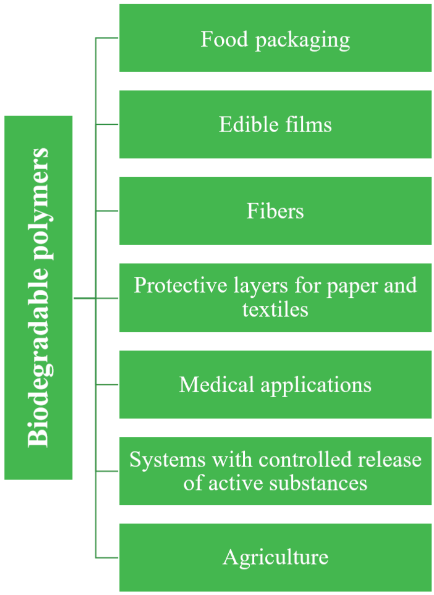

Figure 2. Uses of biodegradable polymers in the industry

Plastic waste is resistant to microbial attack and thus accumulates in large quantities in the soil, thus this resulting waste does not help to fertilize the soil. The best alternative for plastic waste is to use degradable plastic materials. Natural polymers such as starch, wood flour are biodegradable, while most synthetic polymers are not. Additives of high molecular weight plastics, such as plasticizers and reinforcing agents, are susceptible to microbial attack (Maitlo et al., 2022).

Biodegradable biopolymers represent a current field of scientific research of great ecological, scientific and economic importance (Figure 2) (Baranwal et al., 2022). However, the concern for the environmental consequences of products made of such materials, when they end up in landfills after having fulfilled their role, is continuously increasing. In particular, it raises material, disposable problems, such as food packaging. Macromolecules with high molecular mass, containing covalent bonds, are not easily decomposed naturally, under the conditions provided by waste management infrastructures. Polymeric materials manufactured starting from natural biopolymers, such as polysaccharides (such as starch and cellulose), proteins, triglycerides (vegetable oils), generally agricultural products, can be biodegradable and can play a considerable role in solving the environmental problems raised by the use of polymeric food packaging materials (Berradi et al., 2023). Biodegradable polymers can also be obtained by bacterial biosynthesis from natural materials (polysaccharide polyesters), or by chemical synthesis from renewable natural materials (lactic acid polyesters - obtained by fermentation starting from starch). Small products based on synthetic polymers and biopolymers can present a greater or lesser degree of biodegradability (Garrison et al., 2016). Biodegradable materials undergo a decomposition process, resulting in carbon dioxide, methane, water and other organic products, under the enzymatic action of some microorganisms (Figure 3).

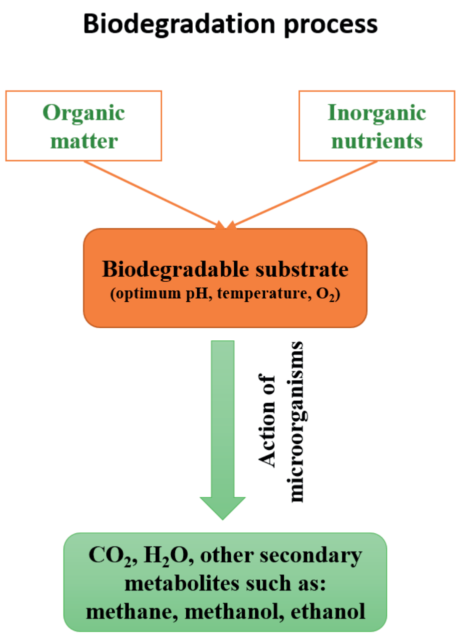

Figure 3. Biodegradation process (own source)

Biodegradable polymers of petrochemical origin

The first researches for their realization were undertaken in the 1970s, consisting in the association of polyethylene with starch or cellulose (Čolnik et al., 2020). By introducing a quantity of 10% starch into a polyethylene matrix, to which 1% catalysts are added to cause the breaking of chemical chains and the consumption of starch by microorganisms, the biological degradation of the plastic material is achieved (Wani et al., 2018). The process was used in the manufacture of polyethylene films for the protection of agricultural seedlings or for bags and packaging, today being abandoned. Recently, a new family of biodegradable polymers containing iron carbamates, nickel and manganese or nickel stearate, as oxidizing agents, were developed (George et al., 2018). Environmentalists were very circumspect about these oxidants, since they actually fragment and not biodegrade the polymers, and the metals that come from the mentioned salts will themselves pollute the environment. The latest research aims to obtain biodegradable materials, through various processes, starting from polymers of petrochemical origin: aliphatic polymers (polycaprolactam, polytetramethylene), polyesters and vinyl polymers. The materials obtained correspond to the norms of environmental protection, but they are expensive (Singh et al., 2014).

Biodegradable polymers of natural origin

They are substances synthesized through biological or chemical processes, starting from natural monomers. This category includes biopolymers, polymers of bacterial origin and synthetic polymers. Biopolymers (natural polymers) are of vegetable and animal origin or are produced by microorganisms (Luckachan & Pillai, 2011). The most important family of natural polymers is that of polysaccharides, which includes starch (derived from potatoes, wheat and corn), cellulose and lignin. Another family of natural polymers is composed up of proteins from oleaginous plants (rapeseed, sunflower, soybean, pea and bean), from cereal bran (wheat gluten), from animal tissues (collagen, gelatine) or from cow's milk (casein) (Gandini et al., 2016).

Polymers of bacterial origin are produced by certain bacteria that accumulate them in the cytoplasm, through fermentation (Rehm, 2010). Examples of polymers of bacterial origin are polyhydroxybutyrate and polyhydroxyvalerate. These polymers are considered semi-biosynthetic and they can also be obtained from some genetically modified plants, in which case they are called biosynthetic (McAdam et al., 2020).

Starch is a cheap material obtained from the processing of corn and other crops. Biodegradation of starch products recycles atmospheric CO_2 produced by starch-producing plants. Starch contains amylase and amylopectin, at rates that vary depending on the starch-producing source. Biodegradable polymers obtained from starch can be produced by mixing them with synthetic polymers (Shrestha & Halley, 2014). Cellulose obtained from chemically modified plants is used in various applications, for example, cellulose acetate is used in many everyday items such as toothbrush handles as well as adhesive tape.

Synthetic polymers are obtained by polymerizing some natural monomers, the best known being polylactide (PLA) which is obtained by bacterial fermentation of lactic acid. This category also includes polyhydroxyalkanoates (PHA) which are polyesters of aliphatic hydroxy acids, polycaprolactone (PCL) and polyglycolide (PGA). The mechanical properties can be improved by combining these polymers, obtaining co-polymers (Nampoothiri et al., 2010).

Table 1. Bio-based food packaging materials – uses and advantages

Biopolymer	Treated food product	Active element	Reference
Soy protein isolate	Bluefin tuna filets	Montmorillonite and clove essential oil	Echeverría et al., 2018
Chitosan and Corn starch	Blueberries	Lemon essential oil and grapefruit seed extract	Bof et al., 2021
Corn starch	Cheese	Green synthesized AgNPs	Ortega et al., 2017
Tapioca starch	Chicken meat	Grape pomace extracts and cellulose nanocrystals	Xu et al., 2018
Polylactic acid	Cottage cheese	Commercial nanoparticles: TiO_2, nano-TiO_2 + nano-Ag	Li et al., 2017
Curdlan + PVA	Pork meat	Thyme essential oil	Zhang et al., 2020
Whey protein	Kasar cheese	Oregano and garlic essential oils	Seydim et al., 2020
Gelatin/Gellan gum	Milk and fish	Red radish anthocyanins	Zhai et al., 2018

Biocompatible polymers

Biocompatible polymers (biopolymers) are polymers present in living organisms (animals, plants, algae) or synthetic polymers of natural origin (PLA, PHA, PHB, PHV, PHBV, PCL, PGA). They are not rejected by the human body and, in general, are biodegradable. Many synthetic polymers of natural origin are obtained through a bacterial fermentation process, using renewable substrates of carbon, carbohydrates and lipids (Chen et al., 2008). There are several hundred types of bacteria that accumulate in cells natural polymers from culture media, polymers can reach 80% of the dry cell mass. They are then extracted from the dried cells by dissolution with organic solvents and then by precipitation in methanol or ethanol.

The applications of biocompatible polymers are found especially in medicine and in the pharmaceutical industry (Koller, 2018). They must have a high chemical resistance, withstand multiple sterilizations (with steam, ethylene oxide or x-rays treatment), without losing their mechanical properties and biocompatibility (Mukherjee et al., 2023). They must also possess very good mechanical properties, especially rigidity, resistance to breaking and durability in order for them to be considered for being used in the food packaging industry (Khatun et al., 2023). A field of great interest for the use of biocompatible polymers is the controlled release of some active substances in the human body or for the use in food packaging materials for the same reason (Westlake et al., 2023). This can be done multiple ways: the active substances are embedded in a core protected by a polymer membrane through which it is diffused into the food packaging system or the human body over a longer period of time or the active substance is embedded in a biodegradable polymer that disappears with the distribution of the active principle in the food packaging system or the human body (Azman et al., 2023).

CONCLUSIONS

Conventional polymers such as polyethylene and polypropylene persist for many years after their disposal in environment. These polymers are unsuitable for applications in the food packaging industry where they are used because they are complicated to recycle and they do not have the capacity to biodegrade. Moreover, plastic materials are contaminated by biological substances, making their physical recycling impractical and undesirable. Due to environmental concerns the food packaging industry is slowly changing the way it uses plastics and other non-biodegradable polymers in the production of packaging materials. Natural biopolymers, such as polysaccharides (starch and cellulose), proteins, triglycerides (vegetable oils) or other agricultural derived products are being studied and used because their biodegradable and compostable proprieties, thus playing an important role in solving the environmental problems raised by the use of polymeric materials. There are several ways biodegradable polymers can be obtained: through bacterial biosynthesis from natural materials (polysaccharide polyesters), or through chemical synthesis from renewable natural materials (lactic acid polyesters obtained by fermentation starting from starch. Bio-based food packaging materials are also very important because they can help extend the shelf=life of food products due to their capacity to be enriched with antimicrobial substances like essential oils, chitosan and many other active substances. Also, researchers have concluded that these novel packaging materials can protect the food product by preventing physical, chemical, and/or microbiological contamination, thus fulfil the function of a barrier against microorganisms, oxygen, humidity, UV and undesirable odours. The uses of these novel bio-based food packaging materials not only are sustainable and advantageous for the reduction of environmental pollution, but also can help producers keep their food products longer on the store shelfs.

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INTEGRATING FRESHWATER SWAN MUSSEL *Anodonta cygnea* IN POLYCULTURE WITH FISH: ESTABLISHING A CONTROLLED ZONE WITHIN THE LOWER SECTION OF A FISH CAGE FARM

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Abstract

Integrated multi-trophic aquaculture (IMTA) presents opportunities for transforming diverse aquaculture-generated waste into revenue streams through the growth of species of economic and biological value. In fresh water, swan mussel Anodonta cygnea (Linnaeus, 1758) exhibit the capacity to purify aquaculture effluent by extracting nutrients contained within it. Also, it represents a value food source for fish, like Black carp, and human consumption. The study aimed to propose an IMTA strategy for freshwater cage fish farming. The feed provided to caged fish is not entirely consumed, serving as the foundation of the food chain for other organisms like swan mussels. To enhance feed efficiency for the fish, which settles at the bottom of the culture ponds where floating fish cages are located, individual net compartments are installed to accommodate the swan mussels. This association yields positive effects across all levels of the food chain by reducing losses and generating a living raw material that falls within the dietary range of other species. Also, through the feeding process, swan mussels filter the water and prevent the undesirable phenomena of decomposition of organic matter and decrease in oxygen concentration, which occur with increasing water temperature. This experiment applies the concept of IMTA by the simultaneous activity of fish and swan mussel farming, which results in minimizing economic losses, maximizing profit and environment protection by cleaning the water.

Key words: *aquaculture, bivalve, cage culture, IMTA, wastes valorisation.*

INTRODUCTION

Romania holds 25% of the total area under fish farming in the European Union, although in 1988, Romania produced 55.000 tons of fish for consumption in fish farming alone, now Romania's domestic production is about 12.500 tonnes of fish in fish farming, 3.500 tons in inland commercial fishing and 8.000 tons in the Black Sea fishery of which 7.500 tons are veined rapa whelk *Rapana venosa* (Valenciennes, 1846), and only 500 tons of fish. In last years, Romania imports over 100,000 tons of fish and fishery products, mainly frozen and in various forms (https://www.fao.org/fishery/en/facp/rou?lang=en). Of this, 15,000 tons are from species also produced domestically in fish farms, including common carp *Cyprinus carpio* (Linnaeus, 1758), Prussian carp *Carassius gibelio* (Bloch, 1782), and Rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) (Neculita & Moga, 2015).

The method of fish farming applied in Romania (Costache et al., 2021a), and in general in Central and Eastern Europe, is the traditional method, in land-based fish farms (Radu et al., 2018; Costache et al., 2021b). However, recent concerns about controlled fish farming have arisen across all regions of the country.

The notable benefits that aquaculture provides to society include: maintaining wetlands and specific microclimates, generating habitats for water-loving plant and animal species, shelters, breeding and nesting sites for birds frequenting areas in the immediate vicinity of water bodies, providing a suitable feeding environment for birds, hydrological regulation of groundwater near aquaculture areas, maintaining local biodiversity (Hanif, 2022), especially in protected areas of the country, and preserving cultural values. Stocking fish farms to maximize resource utilization, including accumulation lakes and any water source that can sustain growing activity, can reduce algal blooms, maintain optimal water chemistry and significantly reduce waste (Costache et al., 2021a; Azhar & Memiş, 2023; Nissar et al., 2023). The predominant fish farming activities in our country include freshwater fish polyculture rearing, both in classical rearing systems and in recirculating systems, floating cages and pens, concrete tanks and controlled systems.

Among the most active preoccupations, worldwide, are increasing aquaculture production with minimal damaging effect on the environment and supplying quality products, with the most reasonable effort involved (FAO, 2020; Arcade et al., 2023). Fishery in our country, as a zootechnical specialization, has focused mainly on the development of technical-scientific methodologies of fish breeding and rearing, creating fish breeds with high productive genetic characteristics (Costache et al., 2018). The trend of increasing productivity per unit area has become more prevalent with the passage of time and the foundation of developed practices (Ahmed et al., 2019; Heydari et al., 2023). The major problem in developing a technology adapted to the new requirements is dimensioning the populations and selecting the most suitable classes of aquatic organisms so that the specific technological consumptions are minimal and the growth rate is upward by introducing extractive, bio-remedial species such as algae, crustaceans etc. into the culture (Ranjbar et al., 2021).

In order to be able to isolate a fish population and grow it in a controlled, more efficient method, it is practiced to use floating fish cages, placed on the surface of a water body (Bucur et al., 2016; Araujo et al., 2022). World-wide, this practice is predominantly applied in open waters (inland and marine). The use of controlled areas such as floating cages has many advantages such as: the possibility of isolating populations by age and species, the possibility of administering feed manually, semi-automatically and automatically, the easy handling of fish material and the reduction of stress during harvesting. In addition to all these advantages, there is also the efficiency of production costs, the possibility of introducing several species of fish, which are not normally compatible in common growing, and the interconnection of several branches of animal husbandry in the fish growing process (Bardach et al., 1972).

Currently, intensive fish culture in cages (with floating nets) is widespread internationally and is indicated as one of the main methods of intensive fish production in the Tropics (Liao et al., 2004; Sangirova et al., 2020). Over the last decade, intensive cage aquaculture in countries such as Brazil, China, for example, has expanding rapidly at an alarming rate, generating substantial additional profits and increasing production and hence export of fish for global consumption. The impact on the aquatic environment created by externalised waste (fish faeces, uneaten feed, bacterial biomass created, destabilisation of the aquatic environment used) from fish farms, especially caged fish farms, is a growing concern worldwide. Increasing the intensity of cage fish farming can lead to harmful effects such as eutrophication and negative effects on water quality. There are several papers that actively seek to show the effect of cage fish farming on the quality of the environment and water (Ntengwe & Edema, 2008; Azevedo et al., 2011; Schenone et al., 2011).

In Romania, fish farming in cages is mainly carried out in small fish farms (earth ponds), but also in water reservoirs. It is essential to control the physical, chemical and biological parameters of water in fish farming technologies. These parameters can greatly affect the survival and growth of fish as well as the development of secondary species such as aquatic plants and crustaceans (Lachi et al., 2008). In addition to the water quality parameters listed above, microorganisms play an important role in the natural cycling of chemical elements inside a water body (Koroleff, 1976). They have a very important function in aquatic ecosystems as they actively participate in the chemical cycling of nutrients. They are also a natural food source for animals, contribute to disease control and can also influence various water quality parameters such as dissolved oxygen, pH and organic matter, ratio of priority elements such as nitrogen, phosphorus, magnesium, fluoride, iodine, ammonia (Moriarty, 1997). The aquaculture in cages can also induce other effects on aquatic ecosystems, including pronounced destabilization of natural habitats (Song et al., 2023) and changes in the structure and dynamics of local organisms, but also trophic changes (Agostinho et al., 2008; Dias et al., 2012; Klinger & Naylor, 2012; Wang et al., 2012). The success of cage aquaculture depends on the physical, chemical and biological characteristics of the water source, the constructive characteristics of the aquatic life ecosystem in which aquaculture is practised, and good nutritional management of the targeted species (Ridler et al., 2007). All these variables in the ponds and the lakes populated with fish are interdependent and require continuous monitoring to avoid the contamination and/or the deterioration of the aquatic habitat. The introduction of bivalve molluscs into freshwater ponds, has led to remediation of the effects of environmental pollution and provided multiple benefits (Khan, 2019; Poznańska-Kakareko et al., 2021).

MATERIALS AND METHODS

The experiment was carried out at SCDP Nucet, in experimental pond number 3 (BE3). In terms of construction techniques, this pond is a classical construction, with water inflow and outflow system, the bottom is composed of alternating layers of soil and other specific sub-strates, and the embankment are simple rein-forced concrete constructions. The design cha-racteristics are presented in Figures 1, and 2.

Figure 1. Experimental pond 3 (B.E.3) (Original)

To minimise the effect of rearing fish in floating cages, a new population was introduced into the food chain of the rearing culture system. This species is popularly referred to as the swan mussel and is a freshwater bivalve mollusc, swan mussel *Anodonta cygnea* (Linnaeus, 1758), and it was introduced to try to remedy the environmental problems and to make the resulting production more efficient (Whitmarsh et al., 2006).

Figure 2. B.E.3 pond - Defining construction element (Original)

The swan mussel *A. cygnea* was introduced because of its ability to biofilter fish effluent water and is also a marker of environmental quality. These aquatic organisms were strategically placed in cages, made of a metal structure to prevent them from floating, and hermetically locked with a fishing net with a mesh diameter of 2 cm (Figure 3).

Figure 3. *A. cygnea* - biometric data recording, before stocking in freshwater pond (Original)

The swan mussels were carefully attached to the construction of the floating cages to allow for movement in tandem with the waves and current on the water.
Stocking fish formulas have been established so that the combination of different species can be complementary and not in competition for feeding. The freshwater swan mussels introduced were taken from another fish pond owned by SCDP Nucet. They entered the pond from their wild habitat (Ilfov stream - this is also the source of water supply to the experimental site studied). The estimated age of the swan mussels, following specific analysis to determine this indicator, it was predominantly in the range of 8-10 years. The size of the swan mussels was variable in the range 8-15 cm long and a variable total weight (shell + body) of 100-170 g.

Data recording

To monitor the physico-chemical parameters of the water, a HANNA JI9829-11042 portable oxygen analyser was used, equipped with special sensors to determine pH, water and air temperature, dissolved oxygen, conductivity and saturation. It is manufactured in Romania and is equipped with sensors for measuring the mentioned parameters, which are calibrated monthly according to the protocol requested by the device.

Laboratory analysis

Chemical analysis of water is carried out by standard methods for determining Ca/Mg ratio, alkalinity, nitrite, nitrate, phosphate, chloride, ammonium, organic matter, pH, total hardness. Finally, the data obtained are transcribed into water analysis reports and these water samples are analysed in the SCDP Nucet laboratory.

Construction characteristics of fish cages and mussel cages

Floating fish cages are technological platforms equipped with anchoring systems (bivalve cages are made of light metal to make it difficult to move the fish cages without holding them in place) consisting of multiple isolated fish rearing modules mounted on a system of floats sized to support the entire construction. The anchoring cages are sized according to the weight of the aggregate, wind force and wave pressure in the pond.
The growth modules have the same dimensions (6 m long and 6 m wide, with a total height of 2 m in the water and 80 cm above water level) and are four by four, in total the system installed in the experimental pond (BE3) contains 8 panels which are separated by a wooden pontoon. Each module is covered with polyester fishing nets with a mesh size of 10 mm and a twine thickness of 2.5 mm, the nets are not constructed with knots and have been pre-treated with a substance to make them more slippery so as not to harm the fish. The floating body is made of HDPE and is interconnected by heating and gluing. Structurally the nets have the following specifications (Figure 4.):

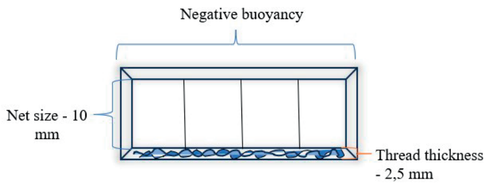

Figure 4. Construction characteristics of floating live-nets and swan mussels cages (Original)

Another important characteristic of net manufacture is that it shouldn't allow fish fins to catch in it or damage it, and nets must be non-toxic and able to tolerate a large temperature variation.
Metal cages have a dual purpose, both to isolate freshwater swan mussels and to serve as an anchor, also preventing the cages from moving over large areas. In terms of dimensions, they are designed to be 70 cm long, 50 cm wide and 30 cm high, resulting in a cage volume of 0.105 m^3. The nets used to cover them are of the same construction as those used in the floating system.

Norms for stocking fish ponds with different fish species

An effective method of stocking floating cages is polyculture, which will minimise antagonistic relationships and maximise the synergies of relationships in the pond. In this way, there will be an increase in the availability of food resources for reared species and a successive improvement in the environmental conditions.

Stocking fish formula of a 70 m^3 cage volume, with majority cyprinids, is presented in Table 1.

Table 1. Population formula for a 70 m3 floating cage in a super-intensive rearing system

Species and age	Floating cage volume (m3)	No. of units	%	Average weight of unit (g/ex)	Quantity (kg)
Carp (1-1+ age)	70	150	30	120	18
Silver carp		80	16	70	5.6
Bighead carp		80	16	70	5.6
Grass carp		80	16	70	5.6
Predatory fish		110	22	50	5.5
Total	70	500	100	Total quantity = 40.3 kg	

The average size of the introduced swan mussels is ~15.5 cm average total shell length, ~7 cm average body height and a weight of 211 g total mass + residual water (internal + external) (Figure 5).

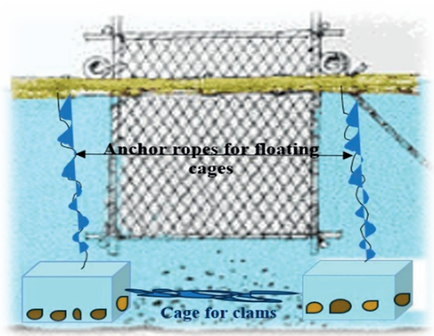

Figure 5. Scheme for anchoring cages to grow swan mussels under floating fish cages (Processed)

Based on these approximate sizes, their distribution over the surface of a cage, in the hypothetical case of occupying the entire surface of the cage base, the estimated number of individuals is 28 swan mussels of identical size (ideal case); however, the total number of swan mussels introduced was 30 ex./cage.

The total number of anchored cages was six cages, distributed in pairs of three cages, tied on the outside of the cage at equal and parallel distances.

Stocking formula of the metal cages with swan mussels, is presented in Table 2.

Table 2. Formula of popular creep cage for swan mussels (*A. cygnea*)

Species ***A. cygnea***	**Cage volume (m^3)**	**No. of units**	**Average weight of unit (g)**	**Average quantity (kg)**
Total cage 1	0.105	30	211	6.300
Total cage 2	0.105	30	206	6.180
Total cage 3	0.105	30	219	6.570
Total cage 4	0.105	30	198	5.940
Total cage 5	0.105	30	201	6.030
Total cage 6	0.105	30	209	6.270

In order to keep the swan mussel cages under the floating fish cages and to avoid distancing them, the two pairs were linked in series and in parallel to follow its exact trajectory. The length of the mooring ropes is designed to minimise the contact between the cage and the surface layer of the pond bottom, so that the system created can cover as short a distance as possible, but it can cross variable areas of the pond so that the process runs smoothly. If it is not possible to migrate the whole assembly, then oxygenation problems and destabilisation of the vital processes of the aquatic life being monitored may appear.

RESULTS AND DISCUSSIONS

The selection of the appropriate aquatic habitat for the development of all the organisms under investigation, was carried out according to several criteria, such as: to present a classical construction in order to improve its natural productivity; the dike delimiting the freshwater pond should be made of cement in order to avoid the risk of blocking the floating cages in the ground; its depth should be suitable for the proper execution of the experiment; it should have a water inflow and outflow installation.

The purpose of the experiment was to introduce swan mussels into a freshwater pond used for polyculture of carp, Asian cyprinids and predators, to demonstrate the benefits of integrating them into the system.

The bivalves were placed in specially designed cages, positioned parallel to the floating fish cages. The rationale for their introduction was to recycle nutrients deposited, on the bottom of the freshwater pond, and to maintain the balance of the ecosystem where the floating cages crossed the water body. The stocking formula were set to be super-intensive in the area of the fish cages and to allow sufficient space for mussel growth.

Water samples were taken from the fish cages before the bivalves were introduced, and after the swan mussels were placed in the specially created spaces. The parameters which were monitored, oxygen and organic matter, underwent variable changes. Throughout the experiment, fish were fed with pelleted feed.

There are a wide range of advantages of integrated fish farming (rearing fish in pens and cages), but there may also be negative precursors of this intensive fish farming, as shown in Table 3.

Table 3. Advantages and disadvantages of integrated multi-trophic cultures in floating cages

Advantages	Disadvantages	Remediation possibilities
The organic waste derived from the diet, rich in macroergic substances, can be used as feed by the fish in the ponds where they are mounted;	This type of integrated fish farming system, based on a high-protein diet, involves feeding a species with a high economic value to make this type of production profitable;	Introducing a fish species with high economic value and obtaining adjacent aquatic crops that can generate profit;
As a consequence of the decomposition of the resulting residues, it has the potential to be recovered by the next food chain in freshwater ponds;	With integrated fish farming, due to the high concentration of accumulated residues, eutrophication of waters can occur;	Introducing into aquaculture a species of living organisms capable of constantly maintaining the trophic and chemical balance of the ecosystem;
Integrated fish rearing in floating cages leads, to a much higher growth of fish material compared to conventional systems and, on the other part, the whole system can be controlled more easily;	For the application of such a rearing process, the need to know the relationships between living organisms and their habitat involves many variables;	Develop perfectly balanced stocking formula rules in terms of species synergy and sustainable exploitation of natural resources;
Stimulates the development of natural feed, thanks to the waste released into the environment from cages and pens.	The possibility to accumulate excessive waste and disturb chemical balances in the water source.	Maintain as stable as possible the chemical equilibrium of the water and the development of active biomass in ponds, where this type of construction is installed.

After the introduction of swan mussels *A. cygnea*, it was observed an increased efficiency, in terms of the consumption of supplementary feed administered, due to the fact that another consumer class with a different feeding behaviour was introduced into the system. Due to the fact that the anchoring system of the cages allowed the "sweeping" of the bottom of the cage, the possibility for swan mussels to feed has been favoured, because these bivalves are sedentary and swim very short distances in their habitat.

By analysing the water and soil samples taken in the SCDP Nucet's own laboratory, it was possible to determine the concentration of organic solids. Due to the displacement of sediments deposited on the bottom of the pond (the water in the pond has an organic substance, containing organic nitrogen or sulphur compounds, humic acid or humates, etc.) there is an improvement of this fish water quality parameter by constant replenishment of the Benthic zone found at the bottom of the pond (Table 4.).

Each lot of water samples analysed (3 samples were collected from the same key points, cage 1, cage 3 and cage 6), it was collected at 14-day intervals, starting with lot 1, which represents time zero for both experimental variants (without swan mussels - variant 1 and with swan mussels - variant 2).

In Table 4, Lot 1 coincides with time zero (T0). Time zero (T0) represents the time when sampling started, day 1, but also day 91 (the first day after the introduction of swan mussels into the specially designed cages). Lot 2 corresponds to the next two weeks (day 14) and so on for a period of 6 months. The six months coincide with the warm growing season for fish.

In the water samples collected from the bottom of the floating cages (20 cm depth), the level of organic matter ($KMnO_4$ – oxidability, expressed in mg/l) averaged 49 mg/l $KMnO_4$ for all batches of samples collected before the introduction of swan mussels into the mussel cages. The same parameter underwent variable changes, with the introduction of freshwater swan mussels into the system, reaching a lower value of 34 mg/l $KMnO_4$.

Table 4. Data obtained by physical-chemical analysis of water samples from floating fish cages

Sample lot/ Characteristics	Oxidability $KMnO_4$ (mg/l)		Dissolved oxygen (mg/l)	
	Without *A. cygnea* - variant 1 (day 1)	With *A. cygnea* - variant 2 (day 91)	Without *A. cygnea* - variant 1 (day 1)	With *A. cygnea* - variant 2 (day 91)
Lot 1/ Feed administration	48	38	5.8	6.1
Lot 2/ Feed administration	50	36	5.4	6.2
Lot 3/ Feed administration	50	36	5.2	5.8
Lot 4/ Feed administration	47	32	6.1	6.5
Lot 5/ Feed administration	49	32	6.6	6.9
Lot 6/ Feed administration	50	31	6.9	7.1

The standard value of this parameter in fish farming waters is in the optimal range 20 - 60 mg/l. The average difference in $KMnO_4$ between the two sets (without swan mussels and with swan mussels) is 14.83 mg/l, lower than the baseline (Figure 6).

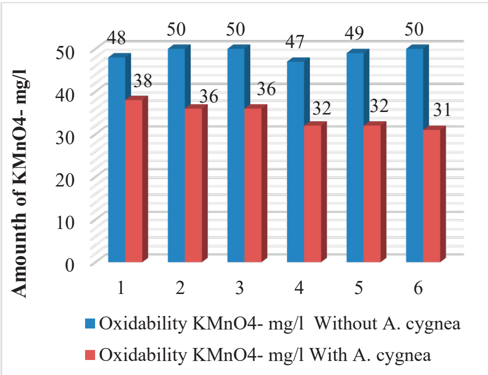

Figure 6. Variation of $KMnO_4$ (mg/l)

The average dissolved oxygen, measured with the HANNA JI9829-11042 portable oxygen meter for all samples before the introduction of swan mussels, was 6 mg/l, and after the addition of swan mussels, the total average changed to 6.43 mg/l. Dissolved oxygen in water increased from an average value of 6 mg/l to 0.43 mg/l (Figure 7).

With the introduction of the bivalve cages, by conducting regular (every 3 days) checks on uneaten food, it was possible to observe a notable reduction in the amount of waste reaching the bottom of the tank, which means that their introduction increases the consumption of additional feed that was distributed to the fish. Periods when more uneaten food was observed after feeding control coincided with changes in wind intensity and increases in daily water temperature. Wind intensity increased the speed with which the cages moved, so that the area covered by the cages was greater than during normal periods. As the contact surface between the cages and the pond bottom has varied, the time spent by the swan mussels for physiological processes (breathing, feeding, excretion) has fluctuated and decreased as well.

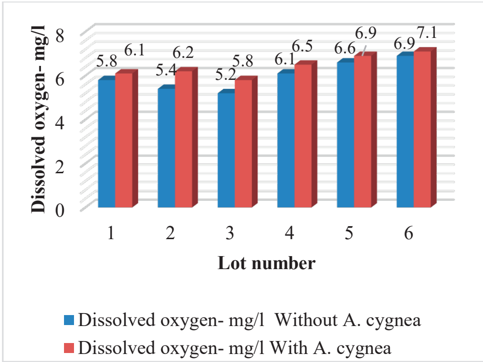

Figure 7. Dissolved Oxygen variation (mg/l)

CONCLUSIONS

The practice of stocking fish and molluscs in cages, as described above, stimulates the application of integrated multi-trophic aquaculture in the current context of circular economy principles. In Romania, the exploitation of freshwater swan mussels is a new chapter, the full benefits of this culture are not yet known and it does not enjoy appropriate attention in terms of popularity. Their introduction at S.C.D.P-Nucet was not intentional, as they ended up in the source water's rearing ponds, together with wild fish that passed through the filters installed on the Ilfov stream. The development considerably of swan mussel *A. cygnea* in freshwater pond was observed during the growing season (April - September). Encouraging results were obtained after biometric measurements, which showed an increasing variation in the total weight of the biomass studied, as well as an increase in total length and girth. Used as biofilters, this edible swan mussel has a valuable performance, is easy to handle and does not require a large investment.

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ANALYSIS OF MEAT QUALITY AND PRODUCTIVITY INDICES IN FISH SPECIES WITH DIFFERENT NUTRITIONAL SPECTRUM

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Abstract

Nutritional behavior and feed intake contribute to fish growth dynamics, productive indices, and meat quality. In this study, productive indices and meat quality were analyzed in three fish species with different nutritional spectrums: a predatory species, the rainbow trout (Oncorhynchus mykiss), an omnivorous species, the wild Danube carp (Cyprinus carpio), and a planktivorous-detritivorous species, the Golden grey mullet (Chelon auratus). A phenotypic characterization of the species was performed (measurements) and body size indices were calculated [Fulton condition factor (K), profile index (Pi), thickness index (Ti), Kiselev quality index (KQi), and fleshiness indices (Ci1-Ci2)], slaughter yields and organs weight ratio and body segments, as well as meat quality. The results show us higher protein values in fish meat from natural environments (Danube carp and Golden grey mullet) due to the more varied nutritional spectrum. Slaughter yields were also better for wild species.

Key words*: body indices, fish meat, nutritional value, slaughter yield.*

INTRODUCTION

Fish meat is an important source of nutritional components, being recommended for daily consumption (Chen et al., 2022). The chemical composition of fish meat differs from one species to another, depending on season, food source, sex, age, and environment (Rasul et al., 2021; Imtiaz et al., 2022; Sandu et al., 2023). The same fish species can have different chemical compositions of the meat depending on muscle segments (epaxial, hypaxial, dorsal, abdominal-intercostal, or caudal peduncle) (Sava et al., 2022). It is important to study the chemical and nutritional composition due to the following aspects: fish is a cheap and easily accessible protein source for the entire world population (Nicolae et al., 2016); polyunsaturated fatty acids have an important role in maintaining human health and in the treatment of some diseases (Ristić-Medić et al., 2013; Carr et al., 2023); and the content of minerals and vitamins is specific to each fish species and differs according to the season.

Statistics show that a cooked portion of 100 g of most types of fish is equivalent to 18-20 g of protein, or a third of the recommended average daily protein intake (Bissih, 2021). Fish protein is of high quality, contains many essential amino acids, and is highly digestible by people of all ages. In fish, there are three main groups of proteins: myofibrillar, sarcoplasmic, and stromal proteins, which constitute 70-80%, 20-30%, and 3%, respectively, of the total proteins in the muscles.

Fish-specific lipids can be divided into two main groups: phospholipids and triglycerides. Phospholipids constitute the integral structure of unit membranes present in cells, being also called structural lipids. Triglycerides have the role of storing energy in fat deposits, being known as storage fats. The latter have unsaturated and saturated monocarboxylic fatty acids in their chemical composition. From the series of saturated fatty acids in fish meat triglycerides, the most representative are considered to be palmitic acid, myristic acid, and stearic acid (Shramko et al., 2020).

The content of minerals and vitamins is specific to each fish species and differs according to season. Fish is an important source of phosphorus, calcium, iron, copper, and selenium. A high iodine content is found in saltwater fish (Sprague et al., 2022). Vitamin B is considered to be one of the most important sources typical of fish meat, and in the case of fatty species, the most common vitamins are A and D (Merdhzanova & Dobreva, 2020).

The purpose of our research was to determine the meat quality (chemical composition) and productive indices of three fish species (rainbow trout - *Oncorhynchus mykiss*; wild Danube carp - *Cyprinus carpio*; mullet - *Chelon auratus*), taking into account their nutritional spectrums: a predatory species, an omnivorous species, and a herbivorous species.

Related to the proposed goal, we established the following research objectives: their phenotypic characterization based on somatic measurements; calculation of body size indices [Fulton K index, profile index (Pi), thickness index (Ti), Kiselev quality index (KQi), fleshiness indices 1 and 2 (Ci1 and Ci2)]; meat quality analysis (water %, dry matter %, crude protein %, crude fat %, ash %); the statistical interpretation of the results obtained and the statement of the conclusions and recommendations).

MATERIALS AND METHODS

This study was organized based on the data regarding the quality of the meat and productive indices from the specialized literature that mentions differences between predatory, omnivorous, and herbivorous fish species. Thus, representative and relatively common species were studied, respecting their spectrum and feeding behaviour.

The phenotypic characterization of the samples studied was based on somatic measurements and weighing: body mass (Bw), total length (Tl), standard length (Sl), commercial length (Cl), maximum height (H), minimum height (h), body depth (Bd), large perimeter (P), small perimeter (p), head length (Hl) and caudal peduncle length (CPl).

Based on the somatic measurements performed, and following the weighing of fish specimens from the three species, body size indices were calculated. These indices provide indications on the productive performance of fish and carcasses (Cocan and Mireşan, 2015). The calculation formulas are as follows:

Fulton condition factor (K)

K = (Bw · 100) / Tl3, where Bw – body mass; Tl – total length;

Profile Index (Pi)

Pi = Sl / H, where Sl – standard length; H – large perimeter;

Thickness Index (Ti)

Ti = (Bd · 100) / Sl, where Bd – body depth; Sl – standard length;

Kiselev Quality Index (KQi)

KQi = Sl / P, where Sl – standard length; P – large perimeter;

Fleshiness index 1 (Ci1)

Ci1 = (Hl · 100) / Sl, where Hl – head length; Sl – standard length;

Fleshiness index 2 (Ci2)

Ci2 = (CPl · 100) / Sl, where CPl – the length of the caudal peduncle; Sl – standard length.

The analyses regarding the chemical composition of the meat consisted of the determination of water content (moisture), dry matter (DM), crude fat (Cf%), crude protein (Cp%), and the determination of mineral substances (ash%). To perform the analyses, the meat from the three species of fish was separated from the bones and the rest of the organs and was mixed (individually each specimen). The dry substance content (DM%) was determined by the percentage difference of the mass of the sample to be analyzed and the water content (moisture%), determined by drying the sample in an oven. Crude protein (Cp%) was determined by the Kjeldahl method. Crude fat (Cf%) was determined by extraction with an organic solvent (petroleum ether) using a Soxhlet apparatus. The determination of mineral substances (Ash%), was done by calcining the samples to be analyzed at a temperature of 600ºC for 5 hours.

RESULTS AND DISCUSSIONS

Following the somatic measurements and weighing, we obtained the results presented in Table 1. The average body weight of the rainbow trout was 236.94±3.37 g, with a minimum value of 197 g and a maximum value of 282.80 g. The average value of the total length was 26.6±0.049 cm, the smallest specimen having 26 cm, and the largest 27.2 cm. Regarding the standard length, its average value was 24.24±0.043 cm, the minimum value was 23.50 cm, and the maximum value was 24.60 cm. The commercial length showed an average value of 18.56±0.066 cm, the minimum being 18 cm and the maximum 19.50 cm. The maximum height, measured in the most developed part of the body, showed an average value of 7.16±0.059 cm, the minimum being 6.5 cm and the maximum 8 cm.

Table 1. Average, minimum and maximum values regarding somatic measurements and body mass of the fish species studied

Parameters	Bw	Tl	Sl	Cl	H	h	P	p	Bd	Hl	CPl
Rainbow trout (*Oncorhynchus mykiss*)											
X±sx	236.94± 3.37	26.6± 0.049	24.24± 0.043	18.56± 0.066	7.16± 0.059	2.78± 0.023	14.78± 0.09	6.7± 0.067	3± 0.031	5.4± 0.053	4.32± 0.033
Min.	197.00	26.00	23.50	18.00	6.50	2.50	13.50	6.00	2.50	4.60	4.00
Max.	282.80	27.20	24.60	19.50	8.00	3.00	16.00	7.50	3.30	6.00	4.70
V%	14.22	1.86	1.79	3.55	8.30	8.20	6.12	10.01	10.27	9.89	7.57
s	33.702	0.495	0.434	0.658	0.594	0.228	0.904	0.671	0.308	0.534	0.327
Common carp (*Cyprinus carpio*)											
X±sx	2810± 79.718	53.18± 0.451	45.72± 0.419	33.7± 0.247	19.12± 0.189	6.64± 0.063	40.02± 0.417	14.6± 0.154	7.88± 0.116	11.74± 0.11	9.2± 0.148
Min.	1700.00	47.10	40.00	29.50	15.80	5.70	34.50	12.40	6.40	10.10	7.50
Max.	3700.00	58.50	50.00	35.50	20.40	7.30	45.30	16.50	9.60	13.10	11.40
V%	28.37	8.47	9.17	7.33	9.89	9.50	10.42	10.57	14.78	9.34	16.14
s	797.183	4.507	4.194	2.469	1.891	0.631	4.170	1.543	1.165	1.097	1.485
Golden grey mullet (*Chelon auratus*)											
X±sx	330.92± 3.876	34.3± 0.164	29.06± 0.113	20.74± 0.184	6.68± 0.054	2.96± 0.022	15.38± 0.08	7.22± 0.03	4.06± 0.039	7.78± 0.018	6.86± 0.074
Min.	273.20	31.50	27.10	18.40	5.90	2.70	14.50	6.90	3.60	7.50	5.70
Max.	368.00	35.60	29.90	23.50	7.20	3.30	16.20	7.50	4.60	7.90	7.70
V%	11.71	4.80	3.90	8.88	8.16	7.40	5.23	4.20	9.63	2.30	10.79
s	38.761	1.645	1.133	1.842	0.545	0.219	0.804	0.303	0.391	0.179	0.740

[1]**Bw** – body weight; **Tl** – total length; **Sl** – standard length; **Cl** – commercial length; **H** – maximum height; **h** – minimum height; **P** – great perimeter; **p** – small perimeter; **Bd** – body depth; **Hl** – head length; **CPl** – caudal peduncle length

The depth of the body presented an average value of 3±0.031 cm, the minimum value obtained being 2.50 cm, and the maximum 3.30 cm. The length of the head (Lcap) and the length of the caudal peduncle (Lped) are not particularly important from a commercial point of view. The average value of the head length was 5.4±0.053 cm, and that of the caudal peduncle was 4.32±0.032 cm.

The average body weight of common carp was 2810±79.718 g, with a minimum value of 1700 g and a maximum value of 3700 g. The average value of the total length was 53.18±0.451 cm, with the smallest specimen at 47.10 cm, and the largest at 58.50 cm. Regarding the standard length, its average value was 45.72±0.419 cm, the minimum value was 40 cm, and the maximum value was 50 cm. The commercial length presented an average value of 33.70±0.247 cm, the minimum being 29.50 cm and the maximum 35.50 cm. The maximum height, measured in the most developed part of the body, presented an average value of 19.12±0.189 cm, the minimum being 15.80 cm and the maximum 20.40 cm. Regarding the minimum height, it presented an average value of 6.64±0.063 cm, a minimum value of 5.70 cm and a maximum value of 7.30 cm. An equally important parameter in terms of fish productivity is the large perimeter. It presented an average value of 40.02±0.417 cm, the minimum value being 34.50 cm and the maximum 45.30 cm. The small perimeter showed an average value of 14.6±0.154 cm, a minimum value of 12.40 cm, and a maximum value of 16.50 cm. The body depth presented a calculated average value of 7.88±0.116 cm, the minimum value obtained being 6.40 cm, and the maximum 9.60 cm. The average value of the head length was 11.47±0.11 cm, and that of the caudal peduncle was 9.20±0.148 cm.

The average body weight of the mullet specimens was 330.92±3.876 g, with a minimum value of 273.20 g and a maximum value of 368.00 g. The average value of the total length was 34.3±0.164 cm, the smallest specimen being 31.50 cm, and the largest 35.60 cm. Regarding the standard length, its average value was 29.06±0.113 cm, the minimum value was 27.10 cm, and the maximum value was 29.90 cm. The commercial length showed an average value of 20.74±0.184 cm, the minimum being 18.40 cm and the maximum 23.50 cm. The maximum height, measured in the most developed part of the body, showed an average value of 6.68±0.054 cm, the minimum being 5.90 cm and the maximum 7.20 cm. Regarding the minimum height, it presented an average value of 2.96±0.022 cm, a minimum value of 2.70 cm and a maximum value of 3.30 cm. The large perimeter presented an average value of 15.38±0.08 cm, the minimum value being 14.50 cm and the maximum 16.20 cm. The small perimeter showed an average value of 7.22±0.03 cm, a minimum value of 6.90 cm and a maximum value of 7.50 cm. The body depth presented a calculated average value of 4.06±0.039 cm, the minimum value obtained being 3.60 cm, and the maximum 4.60 cm. The average value of the head length was 7.78±0.018 cm, and that of the caudal peduncle was 6.86±0.074 cm.

The Fulton condition factor (K) reflects the maintenance status of the fish (Mireșan et al., 2010). The higher the value of this index, the better the maintenance status of the fish. In the case of rainbow trout, it had a value of 1.25 ± 0.012 (V% = 9.39). In the case of common carp, it had a value of 1.82 ± 0.011 (V% = 6.22), and in the case of mullet specimens, it had an average value of 0.89 ± 0.013 (V% = 14.31) (Table 2).

The thickness index (Ig) expresses the width of the musculature (the depth of the body Ac in the most developed region of the body) in relation to the standard length of the fish (Ls). Expressed as a percentage ratio between the depth of the body (Ac) and the standard length of the fish (Ls), the higher value of this index will reflect a better development of the lateral musculature of the fish. The value of the thickness index for rainbow trout was 12.59 ± 0.15 (V% = 11.93), in the case of common carp 17.19 ± 0.14 (V% = 8.12), and in the case of mullet it was 14.00 ± 0.157 (V% = 11.19).

Table 2. Average values and dispersion indices of body size indices for the three fish species studied

Parameters	**K**	**Pi**	**Ti**	**KQi**	**Ci1**	**Ci2**
Rainbow trout (*Oncorhynchus mykiss*)						
X±sx	1.25±0.012	3.4±0.029	12.59±0.15	1.65±0.012	22.28±0.224	17.82±0.12
Min.	1.08	3.04	10.16	1.52	18.93	16.33
Max.	1.41	3.78	14.04	1.82	24.49	19.11
V%	9.39	8.43	11.93	7.09	10.05	6.74
s	0.118	0.287	1.502	0.117	2.239	1.201
Common carp (*Cyprinus carpio*)						
X±sx	1.82±0.011	2.4±0.013	17.19±0.14	1.14±0.004	25.69±0.097	20.04±0.16
Min.	1.63	2.21	16.00	1.09	24.40	18.75
Max.	1.93	2.53	19.51	1.18	26.68	22.80
V%	6.22	5.35	8.12	3.40	3.77	8.00
s	0.113	0.129	1.396	0.039	0.969	1.602
Golden grey mullet (*Chelon auratus*)						
X±sx	0.89±0.013	4.36±0.022	14±0.157	1.89±0.006	26.79±0.064	23.56±0.172
Min.	0.78	4.11	12.16	1.84	26.01	21.03
Max.	1.10	4.59	15.87	1.99	27.68	25.75
V%	14.31	4.97	11.19	3.35	2.41	7.30
s	0.127	0.217	1.567	0.063	0.644	1.721

[1]**K** - Fulton condition factor; **Pi** - profile index; **Ti** - thickness index; **KQi** - Kiselev quality index; **Ci1** - fleshiness index 1; **Ci2** - fleshiness index 2

The profile index (Ip) highlights the body size of the fish and allows individuals of a population to be classified in a certain type of profile (Cocan & Mireșan, 2015). A low-profile index reflects a pronounced convexity of the upper body line, the fish have a chubby appearance, and a chubby spine is correlated with a rich muscle mass in the trunk region. From our evaluations, we obtained a profile index for rainbow trout of 3.4 ± 0.029 (V% = 11.93), 2.4 ± 0.013 (V% = 5.35) for common carp and 4.36 ± 0.022 (V% = 4.97) for mullet.

The quality index (Ica) gives information on the quality of the fish, just by knowing the values of the large body perimeter and the standard length. Specimens with a quality index as low as possible will be retained following the selection processes by breeders, but also in the case of fish destined for slaughter, because specimens with a circumference as large as possible in relation to length are desirable, denoting a rich muscle mass. The value of the quality index for rainbow trout was 1.65 ± 0.012, for common carp 1.14 ± 0.004, and for mullet 1.89 ± 0.006.

The values of the fleshiness indices in the case of rainbow trout were as follows: Ic1 = 22.28 ± 0.224 (V% = 10.05); Ic2 = 17.82 ± 0.17 (V% =

6.74). The values of the fleshiness indices in the case of common carp were as follows: Ic1 = 25.69 ± 0.097 (V% = 3.77); Ic2 = 20.04 ± 0.16 (V% = 8.00), and in the case of mullet Ic1 = 26.79 ± 0.064 (V% = 2.41); Ic2 = 23.56 ± 0.172 (V% = 7.30).
The production of fish meat depends on the production capacity, the quantity, and quality of the administered feed (Nielsen et al., 2002), the environmental conditions (Honcharova et al., 2021; Wang and Mendes, 2022), on the exploitation technologies, and not lastly, on the biological material. Fish meat production can be maximized if optimal environmental conditions are considered. The productive capacity and the economic yield also depend on the anatomical and morphological characteristics of the exploited species, as well as on some physiological peculiarities (Figure 1).

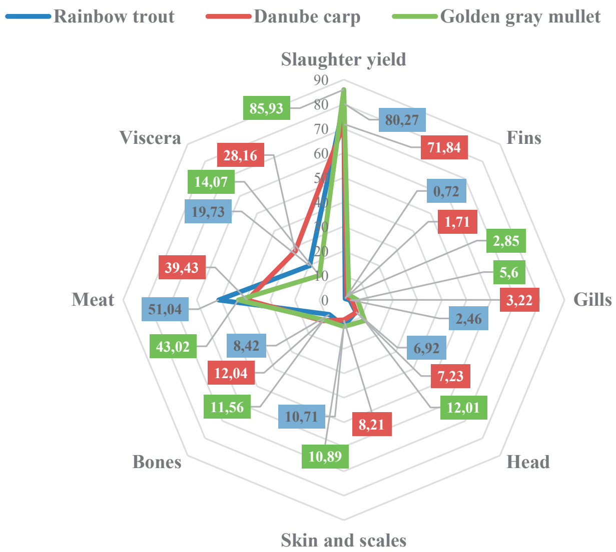

Figure 1. Slaughter yield and weight of viscera, respectively of body segments in the three species studied

To determine the slaughter yield and the weight of the anatomical segments in rainbow trout, 5 specimens with an average body weight of 236.94±3.37 gr were sacrificed. After removing the viscera, the average weight of the carcasses was 200.98 ± 2.58 g and had a slaughter yield of 80.27 ± 0.05%. The viscera weighed 46.75 ± 0.19 g, representing 19.73 ± 0.05% of the initial body weight. The trout head weighed 16.4 ± 0.21 g, representing 6.92 ± 0.04% of the initial body weight. The percentage weight of the fins was only 0.72 ± 0.01% of the initial weight (1.71 ± 0.06 g), while the bones represented 8.42 ± 0.01% (19.95 ± 0.32 g). The skin and scales represented 10.71 ± 0.01% of the initial weight (25.38 ± 0.85 g). The largest percentage share of the initial weight was the somatic musculature (meat), representing 51.04 ± 0.11% of the initial weight (120.93 ± 1.36 g), resulting in a very good yield at slaughter, both in terms of the species of fish, but also compared to other farm animals.
To determine the slaughter yield and the weight of the anatomical segments in common carp, 5 specimens with an average body weight of 2810.80 ± 79.718 g were slaughtered. After removing the viscera, the average weight of the carcasses was 2406.42 ± 68.271 g, with a slaughter yield of 71.84 ± 0.05%. The viscera weighed 791.5 ± 0.19 g, representing 28.16 ± 0.05% of the initial body weight. The carp head weighed 203.12 ± 5.761 g, representing 7.23 ± 0.04% of the initial body weight. The percentage weight of the fins was only 1.71 ± 0.01% of the initial weight (47.98 ± 1.362 g), while the bones represented 12.04 ± 0.01% (338.36 ± 0.32 g). The skin and scales represented 8.21 ± 0.01% of the initial weight

(230.74 ± 6.545 g). The largest percentage of the initial weight was the somatic musculature (meat), representing 39.43 ± 0.11% of the initial weight (1108.5 ± 31.133 g), resulting in a relatively low yield at slaughter, both in terms of the species of fish but also compared to other farm animals.

To determine the yield at slaughter and the weight of the anatomical segments in the mullet, 5 specimens with an average body mass of 330.92±3.876 g were sacrificed. After removing the viscera, the average weight of the carcasses was 299.44 ± 3.26 g, with a slaughter yield of 85.93 ± 0.05%. The viscera weighed 46.56 ± 0.19 g, representing 14.07 ± 0.04% of the initial body weight. The head of the mullets weighed 39.76 ± 0.461 g, representing 12.01 ± 0.06% of the initial body weight. The percentage weight of the fins was only 2.85 ± 0.01% of the initial weight (9.42 ± 0.119 g), while the bones represented 11.56 ± 0.01% (38.26 ± 0.548 g). The skin and scales represented 10.89 ± 0.01% of the initial weight (36.04 ± 1.352 g). The largest percentage of the initial weight was the somatic musculature (meat), representing 43.02 ± 0.12% of the initial weight (142.36 ± 1.306 g), resulting in a relatively good yield, both in terms of fish species and compared to other farm animals.

To determine the chemical composition of rainbow trout meat, 5 specimens of each species were sacrificed. After slaughter, evisceration, decapitation, skinning, and boning, the meat of each specimen was mixed and homogenized using a blender. The following parameters were determined: moisture (moisture), dry matter (DM), crude protein (Cp), crude fat (Cf%), and mineral substances (ash%).

Trout meat is one of the most appreciated varieties of fish meat, basically due to its chemical characteristics, organoleptic and even curative properties. All these aspects derive from the high biological value (content in amino acids and fatty acids), but also from the fact that the growing technology is very well developed and extremely modernized, a fact that leads to the permanent availability of this assortment in specialty stores, in any time of the year.

The results of our study demonstrated an average water content in rainbow trout meat of 76.27 ± 0.14%, with minimum values of 74.02% and a maximum of 77.71%. The average value of the dry matter, calculated by difference, was 23.71 ± 0.142%. Surprisingly, the protein content obtained was slightly below the usual values. Thus, we obtained an average crude protein value of 15.73 ± 0.192%, with a minimum of 14.53% and a maximum value of 19.10%. Normally, the protein level of trout meat is in the range of 17-20%. This fact may be due to the feeds used recently in feeding trout, which contain higher values of lipids, a fact also reflected in the average value of the crude fat obtained (Gb% = 6.92 ± 0.083%). The intake of minerals from rainbow trout meat presented a value of 1.08 ± 0.005%.

Carp is the representative species in terms of cypriniculture (Nicolae et al., 2018). In the case of this species, it should be mentioned that recently, this branch of fish farming is in decline in Romania, the reasons being of an economic nature (the growth period is longer compared to salmonids, large areas of clear water are needed for exploitation, the selling price is relatively low and there is also unfair competition in terms of carp imports, which are found at very low prices due to the subsidies practiced by other countries). In our study, we analyzed the quality of Danube carp meat, a fish caught from the natural environment, and not from aquaculture.

The water content of the carp meat had an average value of 74.44 ± 0.031%, with a minimum of 73.95% and a maximum of 74.72%, the homogeneity (V% = 0.41) resulting precisely from the fact that the fish had the same food resources and the same environmental conditions. The average value of the dry matter was 25.56 ± 0.031%, with a minimum of 25.28% and a maximum value of 26.05%. It is known that the Danube carp has a higher level of protein in the meat than the cultured varieties and breeds. This fact is also demonstrated in this study, the protein level of the meat being 16.06 ± 0.192%, with a minimum of 13.86% and a maximum of 18.80%. Normally, the protein level in aquaculture carp varieties is in the range of 12-15% Pb. The increased level of protein in Danube carp meat is due to the very diverse food supply of the species from natural environments, to the low stress compared to specimens from intensive farms, but also to the adaptations that appeared during the phylogeny.

Regarding the level of lipids in carp meat, there is a distinction between cultured varieties that present consistent deposits of subcutaneous fat, which usually depreciate the organoleptic properties of the meat, and wild varieties (such as the Danube carp) that present a marbled arrangement of fat between muscle fibers, similar to beef. Usually, in carp from aquaculture, the fat level in the meat is in the range of 10-12% Gb. In this study, the average value of fat in carp meat was 8.54 ± 0.199%, with a minimum value of 5.47% and a maximum of 10.58%. The minerals in the Danube carp meat presented an average value of 0.96 ± 0.006%.

Table 3. The chemical composition of the meat of the three species of fish studied

Parameters	Moisture %	Dry Matter %	Crude Protein %	Crude Fat %	Ash %
Rainbow trout (*Oncorhynchus mykiss*)					
X±sx	76.27±0.14	23.71±0.142	15.73±0.192	6.92±0.083	1.08±0.005
Min.	74.02	22.21	14.53	5.88	1.00
Max.	77.71	25.98	19.10	8.16	1.13
V%	1.84	6.00	12.21	12.01	4.50
s	1.402	1.422	1.921	0.831	0.049
Common carp (*Cyprinus carpio*)					
X±sx	74.44±0.031	25.56±0.031	16.06±0.192	8.54±0.199	0.96±0.006
Min.	73.95	25.28	13.86	5.47	0.90
Max.	74.72	26.05	18.80	10.58	1.04
V%	0.41	1.20	11.98	23.33	6.59
s	0.306	0.306	1.925	1.992	0.063
Golden grey mullet (*Chelon auratus*)					
X±sx	75.3±0.233	24.62±0.229	21.05±0.219	2.57±0.077	1.09±0.007
Min.	72.67	21.14	18.63	1.60	1.01
Max.	78.74	27.32	24.19	3.56	1.18
V%	3.09	9.29	10.39	30.15	6.33
s	2.330	2.288	2.187	0.774	0.069

The mullet is widespread in the European and African coasts of the Atlantic Ocean, in the Mediterranean Sea, the Black Sea and the Sea of Azov, but also in their lagoons. In Romania, it is found along the coast, and in the past, it was also found in coastal lakes. Today, attempts are being made to implement technologies for breeding the species in captivity (Niță et al., 2018). It feeds in the sea on algae, benthic, and phytoplanktonic invertebrates, and in the lagoons, it feeds mainly on vegetable detritus.

In our study, we obtained an average water content value of 75.3±0.233%, with a minimum of 72.67% and a maximum of 78.74%. The average value of the dry substance was 24.62±0.229%, with a minimum of 21.14% and a maximum of 27.32%.

There was a very high level of crude protein in the meat, with an average value of 21.05±0.219%, with a minimum of 18.63% and a maximum of 24.19%. These values are clearly higher than those obtained in the case of rainbow trout and common carp, being demonstrated once again that the biological and nutritional value of oceanic and marine fish meat are higher compared to the meat of freshwater fish, respectively those obtained in aquaculture.

Also, the low level of crude fat obtained from mullet meat showed an average value of 2.57±0.077%, with a minimum of 1.60% and a maximum of 3.56%. The importance of fats in fish meat is known. Even if in the case of mullet it had low values, specialized studies mention the high intake of unsaturated fatty acids (monounsaturated MUFA and polyunsaturated PUFA), type Ω3, Ω6, Ω9 and Ω12 (Hedayatifard & Yousefian, 2010). The intake of minerals from the obtained mullet meat was 1.09±0.007%.

Related to our study, it can be observed that the highest average value of water content was obtained for rainbow trout (76.27%), followed by mullet (75.30%), and Danube carp (74.44%). Antagonistically, the content in dry matter presented the following situation in a decreasing manner: Danube carp SU = 25.56%; SU mullet = 24.62%; rainbow trout SU = 23.71%.

The dry matter with the highest possible value is of great importance, being later responsible for the intake of nutrients (proteins, lipids, carbohydrates, minerals). Moreover, a very high water content in meat is not desirable, as the presence of water favours the degradation of the meat and its perishability.

The lowest average value of crude protein was obtained in rainbow trout (Pb% = 15.73%), followed by Danube carp (Pb% = 16.06%) and mullet (Pb% = 21.05). This ranking clearly demonstrates that in terms of nutritional value, mullet is clearly superior to the other two species. Of course, a more complex analysis would require the determination of essential and non-essential amino acid structures, but even so, a substantial protein intake in fish meat is desirable.

Regarding the fats and their average values, Danube carp (Gb% = 8.54%) was in first place, followed by rainbow trout (Gb% = 6.92%), and mullet (Gb% = 2.57%). As mentioned in the case of proteins, it is desirable to have as much fat as possible in fish meat. Similarly to proteins, in the case of fats, research on the structure of saturated and unsaturated fatty acids must be continued (Ljubojevic et al., 2013). Saturated fatty acids are not desirable in very high quantities, but the proportion of monounsaturated fatty acids and especially polyunsaturated ones is particularly important. The latter are involved in various physiological processes, starting from the supply of energy and permeability of membranes to curative and even therapeutic effects (prevention of diseases of the cardiovascular system, liver diseases, prevention and even treatment of various types of cancer, such as rectal or breast cancer) (Salin et al., 2021; Cocan et al., 2010).

In general, minerals in meat are not particularly important, but it must be taken into account that they are involved in numerous physiological processes (Mishra, 2020), and the lack of minerals can lead to various pathological conditions.

CONCLUSIONS

This study demonstrates that there are major differences in terms of meat quality and productive indices between fish species with different nutritional spectrums (herbivores, omnivores and predators). Even under these conditions, it is difficult to make recommendations regarding the consumption of one or other species, because if some species have a high protein content, it is possible, as we have obtained in this study, to have a lower fat content. From a nutritional point of view, a balanced intake of proteins, fats and minerals is recommended. That is why we recommend constant consumption of fish meat and if possible, to diversify the species consumed. Of course, these studies must be continued, because many factors influence the productivity of fish species and the quality of the meat.

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REVIEWING THE POSITIVE IMPACT OF SPIRULINA ON THE HEALTH OF FISH

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Abstract

Aquaculture plays a vital role in global protein production, underscoring the significance of preserving the health and well-being of farmed fish. Spirulina, a blue-green microalgae, is emerging as a promising dietary supplement in aquaculture due to its rich nutritional profile and potential health benefits. This systematic review delves into the existing literature to examine the impact of Spirulina supplementation on fish health. After analysing the selected studies, it was found that various fish species fed with Spirulina-enriched diets showed improved growth, enhanced immune responses, and increased antioxidant capacity. The presence of bioactive compounds, especially phycocyanin, contributes significantly to these health benefits. Despite challenges in determining optimal dosage and addressing environmental considerations, the incorporation of Spirulina into fish diets shows potential for enhancing aquaculture sustainability and productivity. Therefore, this review highlights Spirulina's potential as a beneficial dietary additive in maintaining the general health and welfare of cultured fish.

Key words: *aquaculture, enriched diets, fish health, spirulina.*

INTRODUCTION

Microalgae play a vital role in the aquatic food chain, widely used in aquaculture for the growth of aquatic animals. For example, microalgae serve as a primary food source for the larval stages of many aquatic species and contribute significantly to the overall health and productivity of aquatic ecosystems by improving water quality (Ma & Hu, 2023; Cai et al., 2021; Borowitzka, 1998). The chemical composition of algae provides basic information about their trophic potential. Microalgae are gaining popularity in aquaculture due to their moderate size, high nutritional value, rapid growth, and robust resistance to antioxidants and diseases (Mishra et al., 2022; Habib et al., 2008; Brown et al., 1997).

Algae, as the earliest life forms on Earth, reproduce independently, providing nutrients for the growth of other producers and the next trophic levels. Besides this, they also generate oxygen as a byproduct of their growth, contributing to around 70% of the Earth's free oxygen (Tietze, 2004).

Spirulina (*Arthrospira platensis* Gomont, 1892), a green-blue microalgae, is a symbiotic, multicellular, filamentous organism associated with nitrogen-fixing bacteria. Its unique features include the pigment phycocyanin for photosynthesis, giving it a distinct blue colour. Spirulina's reproduction involves binary fission, and its spiral configuration forms floating mats. It appeared approximately 3.6 billion years ago, utilizing dissolved carbon dioxide present in seawater as a source of nutrients (Vo et al., 2015).

Spirulina has been used therapeutically since the 8th century, initially discovered and utilized by ancient African civilizations and later by Aztecs in Central America (Ciferri, 1983).

Botanically classified as microalgae in the class Cyanophyceae, Spirulina's prokaryotic structure leads bacteriologists to categorize it as bacteria. Its morphological plasticity is influenced by genetic changes, environmental factors, and physico-chemical conditions (Richmond, 2004; Vonshak, 1997). Spirulina is rich in nutrients that transform this alga into a valuable food source and through the years, extensive studies on biochemical composition, safety consumption, physiological effects of enriched diets on animal organisms, and toxicity testing were carried out and proved that Spirulina is a useful feed ingredient for both human and animal consumption (Anvar & Nowruzi, 2021; Habib et al., 2008; Becker, 2007; Vonshak, 1997; Belay et al., 1993).

MATERIALS AND METHODS

In this review, we conducted a comprehensive literature search to gather and analyse existing research on the positive impacts of Spirulina on the health of fish. The primary databases used for the search included Google Scholar, Web of Science and Scopus. The search terms included combinations of keywords such as "Spirulina", "fish health", "aquaculture", "immune response", "growth performance". Inclusion criteria were set to encompass the relevant peer-reviewed articles and reviews. Studies specifically addressing the effects of Spirulina supplementation on various health parameters of fish, such as growth performance, immune response, and overall health were included.

Articles were first screened by title and abstract for relevance, and then full texts were reviewed for detailed information. Data were extracted and synthesized to provide a comprehensive understanding of the current state of research on the topic.

RESULTS AND DISCUSSIONS

Nutritional composition and active compounds of spirulina

Spirulina has captivated the attention of specialists in the field because of its essential biochemical compounds, surpassing many other microalgae species that have been studied (Henrikson, 2009). Spirulina can be easily and cost-effectively cultivated using low-cost culture media. It grows in water, can be harvested and processed easily, and has a significantly high content of macro and micronutrients. Spirulina has been commercially cultivated since the 1960s in various countries worldwide due to its high nutritional content, including high-quality proteins, carbohydrates, amino acids, vitamins, minerals, essential fatty acids, and other bioactive compounds like pigments (β-carotene) and phenolic acids (Vonshak, 1997; Becker, 1994). This composition (Figure 1) varies based on factors such as the source, culture conditions, and production season (Grosshagauer et al., 2020; Falquet, 2017; da Rosa et al., 2016; Vernès et al., 2015; Babadzhanov et al., 2004; Tokuşoglu, 2003; Phang et al., 2000). The 60-70% high protein content is much higher than that found in eggs, meat, milk, soybeans, or other cereals (Belay et al., 2008; Becker, 2007). It is also rich in B-group vitamins such as vitamin B12 (8 ppm) and provitamin A (0.2%), minerals like iron (0.1%), and polyunsaturated fatty acids representing 30% of total lipids, especially ω-6 fatty acids (up to 29.4-31.5% of total fatty acids), linoleic acid, stearidonic acid, arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid (El-Samragy, 2012; Habib et al., 2008). The presence of all essential amino acids constitutes 47% of the total protein, and among all essential amino acids present, valine, leucine, and isoleucine have the highest value (Belay et al., 2008; Becker, 2007). The cellular wall of Spirulina contains polysaccharides with an 86% digestibility, facilitating assimilation by the animal organism (Becker, 1994).

Spirulina is not only rich in nutrients but also serves as a source of phytopigments, including chlorophyll, carotenoids, and phycobiliproteins. These pigments play a vital role in capturing light within Spirulina. In 2013, Kuddus et al. found that the phycobiliproteins extracted from Spirulina are phycocyanin (C-PC), allophycocyanin (A-PC), and phycoerythrin (PE). Later, it was determined that phycocyanin represented up to 50% of phycobiliprotein content (Li et al., 2020).

Due to this valuable composition of useful nutrients, Spirulina became widely a nutritional supplement suitable for animal feed and humans, too (Kim, 2013; Qureshi et al., 1996; Glombitza & Koch, 1989).

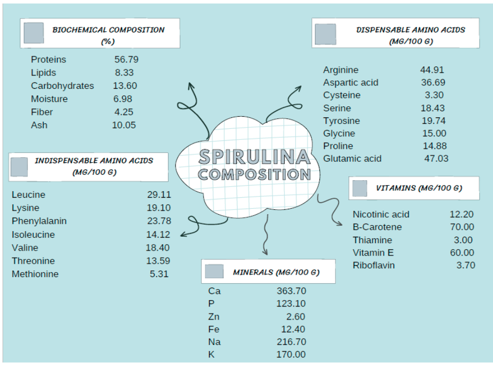

Figure 1. The composition of Spirulina and its bioactive constituents (after El-Moataaz et al., 2019)

Therefore, from the perspective of the biochemical composition, it has been proved that Spirulina is an excellent source of beneficial nutrients and healthful substances, as well as a good source of energy, making it a suitable addition to feed formulations.

Contribution of spirulina to human health

Global development of the Spirulina market primarily involves the utilization of dried whole Spirulina biomass, serving as a healthy dietary supplement for animals and humans, with the assumption that its consumption may promote, prevent, aid, or cure common diseases and malnutrition.

Since the 1980s, there have been found applications of Spirulina in healthy nutrition, animal feed, and biochemical products (Becker, 1988; Borowitzka, 1988).

Numerous commercial products contain Spirulina biomass, Spirulina extracts or its active ingredients and that could be included as additives in animal feed, health-improvement products for animals, natural food and cosmetic colourants, as well as purified biomolecules for medicine and biotechnology (Henrikson, 2009).

Further investigations on the bioactive properties of Spirulina (Teimouri et al., 2016; Kim et al., 2013; Andrews et al., 2011; Promya & Chitmanat, 2011) have highlighted improvements for both health and stress resistance of the organism, reinforcing the idea of its use in animal feed as a functional additive.

Due to its high iron content, Spirulina increases iron absorption by 60% compared to the regular iron supplement. Moreover, studies have indicated anaemia correction in rats and potential benefits in conditions such as arthritis, heart diseases, obesity, and zinc deficiency (Henrikson, 2009).

A comparative experiment on mice specimens treated with Spirulina-derived carotenoids and others treated with synthetic β-carotene demonstrated the superior hepatoprotective effect of Spirulina's bioactive compounds (Chidambara Murthy et al., 2005).

Over time, Spirulina has revealed a wide range of physiological responses in animal organisms, including the substantial improvement of the immune system, with implications extending to human practices.

Therefore, Spirulina has been used in healthy food products such as soups, sauces, pasta, snacks, and instant drinks to enhance their nutritional value. From this point of view, studies have indicated positive results in treating severe malnutrition in children and in reducing blood glucose levels (Vonshak, 1997).

Other clinical studies (Habib et al., 2008) have highlighted multiple benefits of Spirulina, demonstrating the effectiveness of Spirulina capsules in reducing blood lipid levels and decreasing the white blood cell count in patients undergoing radiation and chemotherapy treatments. In support of this finding, investigations on mice exposed to lethal doses of radiation recorded increased survival rates due to the enhancement of the immune system in the specimens with Spirulina administration. Also, incorporated as an effective supplement in human patients' diet, Spirulina has been found to reduce blood lipid levels, enhance fatigue resistance, and elevate the levels of immunoglobulin A (IgA) and immunoglobulin M (IgM) in athletes (Habib et al., 2008). In a vitro study, Spirulina's phycocyanin has also demonstrated the ability to inhibit cells' growth in leukaemia (Liu et al., 2000).

Recently, Anvar & Nowruzi (2021), in their review, indicated that Spirulina was tested as a remedy for various diseases and a daily source of nutrients, being a promising healthy ingredient for the food industry in human nutrition. The authors considered Spirulina as a superfood and an optimal nutritional source for combating malnutrition.

The impact of spirulina in the aquaculture industry

Even from the 1960s, due to simultaneous trends of increasing fish-based product consumption and declining of fish global captures from natural waters, finding alternative protein sources for fish meal, whether plant-based or animal-based, has become essential for preparing balanced fish feed (Burel & Kaushik, 1965). Gladue & Maxey (1994) explored the idea of replacing fishmeal proteins with microalgae as a main protein source in artificial fish feed formulations in aquaculture. Over time, due to continuously decreasing fishmeal supply and rising costs, various algae and plants have been tested as alternative protein sources, with the converged purpose of enhancing the fish meat's colour, flavour, and quality, too (Becker, 2007).
In recent years, aquaculture researchers have focused on the nutritional use of Spirulina as a substitute for fishmeal or as functional feed for aquatic organisms (Table 1). This approach has proved beneficial apport to produce omnivorous and herbivorous fish species that naturally include algae in their diet (Rosas et al., 2019).
A study conducted by Teimouri et al. (2013), investigating various diets of *Spirulina platensis* at 0, 2.5, 5, 7.5, and 10%, alongside synthetic astaxanthin (50 mg), assessed their impact on skin and fillet pigment, as well as on the growth performance of rainbow trout (*Oncorhynchus mykiss*). Their findings revealed that the most significant influence of carotenoids on skin and fillet occurred at a 10% incorporation of *Spirulina platensis* into the fish diet. Additionally, it was demonstrated that Spirulina can replace synthetic astaxanthin in rainbow trout diets.
In terms of cost reduction in fish feed, El-Sheekh et al. (2014) found that incorporating Spirulina flour at a 75% inclusion rate in red tilapia feed resulted in reduced feeding expenses (cost/kg of feed), together with an increased profit index. Furthermore, Nakano & Wiegertjes (2020) have reported that the absence of cellulose from Spirulina's cell structure makes it easily digestible, thereby enhancing feeding appetite. This improvement in food intake and nutrient digestibility contributes to the fish's health enhancement (Figure 2) and the strengthening of their defence against infections due to reduced stress levels.

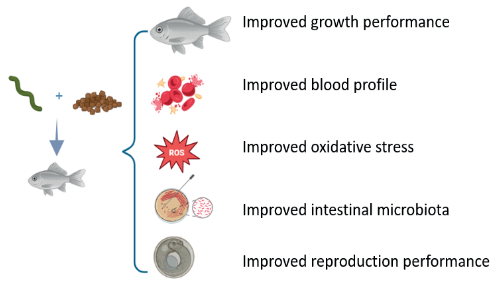

Figure 2. Effects of Spirulina on fish health (after Ragaza et al., 2020)

Table 1. Recent studies exploring the utilization of Spirulina as a supplement in fish feed

Fish species	Proportion of fishmeal or ingredient substituted in feed formulations by Spirulina	Impacts of substituting with Spirulina	References
Nile tilapia (*Oreochromis niloticus)*	0.2% inclusion	↓oxidative stress, ↓tissue damage (liver, kidney, spleen) caused by florfenicol and/or bacterial infection	Abu-Zahra et al. (2024)
Nile tilapia (*Oreochromis niloticus*)	1%, 2% & 3% inclusion	↑growth performance, ↑gene expression, ↑biochemical parameters	Abozaid et al. (2023)
Nile tilapia (*Oreochromis niloticus*)	0.2% & 0.4% inclusion	↑protection against imidacloprid toxicity, ↑growth, ↑haemato-biochemical parameters, ↑oxidant/antioxidant balance, ↑immunity	Abdel-Tawwab et al. (2021)
Nile tilapia (*Oreochromis niloticus*)	15% inclusion	↑fish body protein levels, ↑immune response against *A. hydrophila* infection, ↓oxidative stress,↑enzymatic antioxidants, ↓MDA levels, ↓histopathological changes	El-Habashi et al (2019)
Nile tilapia (*Oreochromis niloticus*)	5–50% inclusion	↑growth, ↑tissue characteristics	Bin Dohaish et al. (2018)
Nile tilapia (*Oreochromis niloticus*)	0.5% & 1% supplementation	↑antioxidant status (hepatic, renal) against diazinon toxicity	Abdelkhalek et al. (2017)
Nile tilapia (*Oreochromis niloticus*)	0%, 30%, 45%, 60% & 75% replacement	↓blood triglyceride levels, ↑productive performance in 30% group	Velasquez et al. (2016)
Nile tilapia (*Oreochromis niloticus*)	up to 1% supplementation	↑ blood serum defences, ↓lipid peroxidation	Abdelkhalet et al. (2015)
Nile tilapia (*Oreochromis niloticus*)	up to 10% supplementation	↑serum antioxidant activities, ↑growth, ↑resistance against *V. algynoliticus*	Abdel-Latif and Riad (2014)
Nile tilapia (*Oreochromis niloticus*)	0.5-2% supplementation	↑health of fish, ↑tissue protection, ↑antioxidant capacity	Ibrahem et al., (2013)
Tilapia (*Oreochromis* sp.)	Up to 43% replacement	↓feed conversion ratio (FCR) in Spirulina groups compared to	Hussein et al.,

Fish species	Proportion of fishmeal or ingredient substituted in feed formulations by Spirulina	Impacts of substituting with Spirulina	References
larvae or juveniles		control diet (corn-gluten meal)	(2013)
Hybrid red tilapia *(Oreochromis niloticus x Oreochromis mossambicus*)	75% replacement	↑growth performance, ↑feed efficiency, ↑ immunity capacity	El-Sheekh et al. (2014)
Rainbow trout (*Oncorhynchus mykiss*) juvenile	250, 500, 1000 & 2500 mg supplementation	↑growth performance, ↑survival rate, ↑FCR, in 250 mg extract of Spirulina group	Kermani et al. (2020)
Rainbow trout (*Oncorhynchus mykiss*)	2.5% & 5% supplementation	↑histological parameters, ↑immune-related gene expression in mucosal tissues and fish resistance against *Y. ruckeri*	Sheikhzadeh et al. (2019)
Rainbow trout (*Oncorhynchus mykiss*)	up to 10% supplementation	↓oxidative stress, ↑increased antioxidant level	Teimouri et al. (2019)
Rainbow trout (*Oncorhynchus mykiss*)	10% supplementation	↑red and white blood counts, ↑haemoglobin, ↑total protein, ↑albumin	Yeganeh et al. (2015)
Rainbow trout (*Oncorhynchus mykiss*)	50 g and 100 g/kg supplementation	↑muscle quality, ↓lipid accumulation, ↑polyunsaturated fatty acids (PUFAs) content (natural antioxidant), ↓reduces lipid peroxidation, ↓postmortem deterioration, ↑human health indicators	Teimouri et al. (2015)
Rainbow trout (*Oncorhynchus mykiss*)	7.5% replacement	↑weight gain	Teimouri et al. (2013)
Caspian brown trout (*Salmo trutta caspius*)	6% & 8% supplementation	↑growth, ↑carcass composition, ↑colouration	Roohani et al. (2019)
Common carp (*Cyprinus carpio*)	10mg/kg supplementation	↑haematological profile, ↑biochemical blood status, ↑growth performance	Ahmed et al. (2023)
Common carp (*Cyprinus carpio*)	0.1, 0.3 & 0.5 supplementation	↑growth performance	Abdulrahman (2014)
Gibel carp (*Carassius auratus*)	up to 100% replacement	↑antioxidant activities, ↑immune system	Cao et al. (2018)
Grass Carp juvenile *(Ctenopharyngodon idella*)	up to 5% supplementation	↑growth performance, ↑antioxidant status, ↑digestive enzyme activity, ↑innate immune status	Faheem et al. (2022)
Persian sturgeon (*Acipenser persicus*)	2.5%, 5% & 7.5% supplementation	↑haematological status, ↑artificial reproduction efficiency in the group with 7.5% Spirulina	Akhoundian et al (2023)
Beluga Sturgeon (*Huso huso*)	up to 10% inclusion	↑growth performance, ↑immune response, ↑disease resistance, ↑ digestive enzyme activity	Adel et al. (2016)
Sabah giant grouper	5% enriched diet	↑growth performance, ↑intestinal microbial function	Man et al. (2020)
Oscar fish (*Astronotus ocellatus*)	55 g/kg supplementation	↑growth performance, ↑feeding parameters, ↑protease activity, ↑ protein content, ↓fat content, ↑immune biochemical status, ↑blood profile, ↑digestive enzyme activities, ↑overall pigmentation	Mohammadiazarm et al. (2021)
Parrot fish (*Oplegnathus fasciatus*)	5% replacement of protein	↑weight gain, ↑protein efficiency ratios, ↑feed intake, ↓feed conversion ratios compared to the control diet (fishmeal)	Kim et al. (2013)

Integration of spirulina in fish feed to boost the immune system and enhance growth performance

The usual ingredients in aquaculture feedstuff are seeds, cereals, and different animal by-products meals which are considered costly components. Therefore, the feeding costs are seen to reach, in general, 60-70% of the operational costs in intensive and semi-intensive aquaculture systems (Singh, 2006). Long-time research has been focused on replacing expensive components, either in part or entirely, with other products. For example, extensive investigations have been conducted to assess various alternative protein sources as partial or complete replacements for fishmeal in different fish species. The examined references have indicated that supplementing fish diets with additives like Spirulina involves multiple nuances related to their targeted effects on various physiological aspects.

Adel et al. (2016) have emphasized Spirulina's ability to improve growth parameters, with notable effects observed, especially at a 10% inclusion rate in great sturgeon's diet. These results regarding enhanced growth, increased activity of digestive enzymes, and improved immune response have contributed to heightening the organism's resistance to bacterial disease. In 2019, another study conducted by El-Habashi et al. obtained similar findings on Nile tilapia. They observed that the supplementation of dried microalgae significantly enhanced fish body protein and immune response against specific infections, while also improving the oxidative stress markers and mitigating histopathological changes.

Recently, Abozaid et al. (2023) investigated the optimal levels of Spirulina incorporation in Nile tilapia diets and found significant enhancements in growth performance, gene expression, and biochemical parameters at a 1% inclusion rate. Nevertheless, they advised against exceeding this optimal level due to potential adverse effects on certain physiological aspects.

Further, Abu-Zahra et al. (2024) revealed the synergistic impact of Spirulina and antibiotics, highlighting the potential risks associated with antibiotic overuse in aquaculture. They recommended for use of alternative additives, such as immunostimulants, in disease treatments to minimize the emerging environmental hazards and bacterial resistance.

Research on rainbow trout (Teimouri et al., 2019; Sheikhzadeh et al., 2019) recorded the antioxidant and immunomodulatory effects of Spirulina, showcasing its potential as a beneficial dietary supplement for fish farming.

The positive effects of Spirulina supplementation on growth performance and stress resistance in rainbow trout were also studied by Kermani (2020).

In the case of grass carp, adding Spirulina up to 5% improved growth performance, antioxidant levels, digestive enzymes, and innate immune indicators (Faheem et al., 2022).

In a recent study, Akhoundian et al. (2023) found notable physiological benefits of incorporating Spirulina into the diet of Persian sturgeon fry. However, they also emphasized the need for further research before implementing Spirulina enriched diet commercially in sturgeon culture.

Integration of spirulina in fish feed to improve fish haematological status and oxidative stress

Generally, fish are more susceptible to diseases when they experience stress triggered by adverse environmental conditions, poor management practices, or a reaction to the limited availability of essential nutrients in their diet (Oliva-Teles et al., 2015).

The evaluation of haematological status is an important and accessible tool in determining the health and wellness of fish. It has been demonstrated that the characteristics of the haematologic indicators have a strong correlation with the animal's reaction to its environment, indicating that some influence may be exerted on histological characteristics by the rearing conditions (Gabriel et al., 2004). These biomarkers offer valuable insights into the fish's circulatory system and how they react to environmental and stress stimuli. Through the analysis of haematological and biochemical blood parameters, it can be identified conditions like anaemia, inflammation, or infections, thereby facilitating health monitoring and the implementation of prophylactic/treatment strategies to enhance the living conditions of fish in both aquaculture and natural settings. (Lataretu, 2013).

The protective efficacy of *Spirulina platensis* against ethidium bromide toxicity in tilapia fish (*Oreochromis niloticus*) was investigated by Abdullah et al. (2024) and they observed that most of the histological and histochemical parameters have returned to baseline levels following *Spirulina platensis* supplementation. These results reinforced previous findings drawn by Sayed et al. (2015; 2017) attributed to *Spirulina platensis*' bioactive compounds, including C-phycocyanin, provitamin A (β-carotene), minerals, vitamins, proteins, carbohydrates, and lipids, which are possessing antioxidant and anti-inflammatory properties, helpful in maintaining the membranes' structural integrity in liver and kidney by scavenging free radicals (Salah El-Din et al., 2021).

The presence of specific pesticides in the water resulted from agricultural practices exposes fish to oxidative stress, causing significant harm to their health. For example, common carp (*Cyprinus carpio*) exposed to atrazine (428 μg/L) and Spirulina (1%), either separately or in combination, for 40 days, demonstrated a significant increase in lipid and DNA oxidative damage markers, and a significant decrease in antioxidant biomarkers. However, the addition of 1% Spirulina to the diet led to a significant decrease in hepatotoxic and inflammatory effects induced by atrazine-induced oxidative stress (Toughan et al., 2018).

Abdelkhalek et al. (2017) observed the protective effects of Spirulina against intoxication induced by certain chemicals and pesticides in Nile tilapia, attributing this to its antioxidant properties capable of mitigating oxidative damage.

High protective effects against toxicity were observed by Abdel-Tawwab (2021) in Nile tilapia fed with Spirulina, as well as the improvement of various physiological parameters.

In the study conducted by Adel et al. (2016) related to the beneficial addition of Spirulina into the diet of the great sturgeon (*Huso huso*), there has been observed a decrease in triglyceride values in comparison with the control group, an increase in haemoglobin concentration, haematocrit value, and neutrophil count, beside the other physiological responses previously mentioned above.

A scientific investigation was undertaken to assess the immunostimulatory impact of a combination of Spirulina and a β-glucan additive (MacroGard®) in *Acipenser stellatus*. The experimental protocol involved feeding the fish with a diet comprising 3% of their body weight, containing 0.1% MacroGard® and 0.5% Spirulina, over 12 weeks. Substantial enhancements in both body metrics and haematological parameters were observed following this feeding regime (Salehi-Farsani et al., 2016).

CONCLUSIONS

In summary, the research underscores the benefits of integrating Spirulina into fish diets, highlighting its potential to enhance growth, support stress resilience, and reinforce immune function. These findings suggest that Spirulina supplementation offers protection against various stressors such as bacterial infections and chemical toxins. However, careful consideration of optimal dosage and avoidance of over-supplementation are imperative to prevent adverse effects on fish health. Consequently, Spirulina emerges as a promising stimulant in aquaculture, for commercial application. Nevertheless, more investigations are necessary to elucidate the underlying mechanisms and ensure the efficacy and responsible utilisation of this technology in fish farming. Overall, Spirulina represents a valuable tool in mitigating the impact of environmental stressors and promoting the health and stress resilience of fish, particularly in aquaculture conditions. where the feeding management allows custom-enriched diets with this alga.

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KRILL OIL SUPPLEMENTATION AMELIORATES FRUCTOSE-INDUCED HYPERTRIGLYCERIDEMIA IN *Carassius auratus calico*

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Abstract

Fructose is a highly lipogenic sugar and excessive fructose intake stimulates endogenous glucose production and lipid synthesis in the liver. Therefore, the present study aims to investigate the amelioration of liver impairment induced by high fructose dietary doses following dietary supplementation with krill oil. 45 exemplars of Carassius auratus calico weighing 150 grams, were randomly distributed into three experimental diets including V1 - Control, fish fed with a normal diet, V2 - fish fed with 1% fructose, V3 - fish fed with 2% fructose, respectively, at the end of 90 days: V1 - Control, fish fed with a normal diet, V2 - fish fed with 1% krill oil, V3 - fish fed with 2% krill oil. The blood tissues were collected to analyze hematological parameters (red blood cell counts (RBCc, x 106/µl), the hematocrit (PCV, %), hemoglobin concentrations (Hb, gl/dl,) HEM, VEM, CHEM and serum biochemistry parameters (GLU, TRIG, ALT and AST). In addition, the organosomatic indices (HIS, VSI) and proximate composition (water, lipid, ash, and protein content) were analyzed. No differences between the control and treated fish from the two experimental diets were observed in hematological parameters. After 90 days fructose-treated fish showed increased AST and ALT levels. Our results showed that daily krill oil supplementation in the Carassius auratus calico prevented fructose-induced hypertriglyceridemia.

***Key words**: Aquaculture krill oil, hematological and serum biochemistry parameters, organosomatic indices.*

INTRODUCTION

The Goldfish (*Carassius auratus calico*) belongs to the family *Cyprinidae* and is one of the most popular freshwater ornamental fish in the world, including in Romania. Being omnivorous, goldfish consume invertebrates and zooplankton from natural water bodies. However, goldfish are commonly fed high-quality granulated or pelleted feeds in artificial breeding systems (Hafeez-ur-Rehman et al., 2015).

Because more than half of the operational costs of ornamental species are associated with food costs, feed is an important factor that can affect fish welfare, growth, and profitability (Jamu & Ayinla, 2013; Fry et al., 2018). The goldfish, a modified variety of *Cyprinus carpio*, could be used as an ideal model for nutritional studies in larval and juvenile cyprinids. Carbohydrates such as glucose and fructose are the least expensive sources of energy for fish diets. Long-term consumption of diets high in carbohydrates leads to a significant accumulation of fat in the serum and liver. This is because carbohydrates can be converted into lipids through a process known as lipogenesis. The omnivorous fish have a greater ability to convert carbohydrates into lipids, as demonstrated in studies on gibel carp (*Carassius gibelio*) (Li et al., 2019).

Despite generally exhibiting higher plasma insulin levels than mammals (Moon, 2001), fish display lower insulin sensitivity and are classified as glucose intolerant. This characteristic makes fish more susceptible to hyperglycemia, which is linked to various metabolic diseases in fish (Prisingkorn et al., 2017; Goessling & Sadler, 2015). Herbivorous and omnivorous fish utilize carbohydrates more efficiently than carnivorous fish due to their more developed digestive system, enzyme activity of glucose metabolism, and complex hormone regulation (Legate et al., 2001). Hyperglycemia contributes to lipid deposition in fish, as Luo et al. (2020) reported.

The effect of high fructose levels on hepatopancreatic function in goldfish has not been studied. In the case of diets with high levels of carbohydrates, functional ingredients are sought to reduce their negative effects. In this context, Krill can be a good candidate for several reasons. Antarctic krill (*Euphausia superba*) may represent the largest biomass of a single species worldwide, and it is a promising ingredient for use in fish feeds. Krill has a balanced amino acid profile, and krill oil contains the essential nutrient, choline, and an antioxidant, astaxanthin (Xie et al., 2019; Bengtson et al., 2014). In addition, Krill oil serves as a valuable alternative source of n-3 polyunsaturated fatty acids (PUFAs), containing a substantial content (30-65%) of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). (Hansen et al., 2011).

Within the aquaculture sector, krill has primarily been investigated as a protein source (Choi et al., 2020; Mørkøre et al., 2020; Olsen et al., 2006), and its utilization for pigmenting fish flesh with carotenoids has also been explored (Arai et al., 1987). However, some research specifically delves into the potential of krill oil (KO) as a functional ingredient to manage stress and promote health in intensively cultured fish (Li et al., 2021). The supplementation with 5 g/kg feed krill oil of the carp diet has been found to enhance growth performance, with no observed alterations in the proximate composition of the meat (Năstac et al., 2023). KO feeding also reduced oxidative damage in the liver, with a decrease in malondialdehyde content and an increase in total antioxidant capacity, thus improving the lysozyme activity and immune status of the carp fingerlings held under different stocking densities (Năstac et al., 2023).

Therefore, the present study aims to investigate the amelioration of liver impairment induced by high fructose dietary doses following dietary supplementation with krill oil.

MATERIALS AND METHODS

Experimental Design

Phase I. 90 Fingerlings of *Carassius auratus calico,* with an individual mean weight of 3.33 ± 0.09 g, were randomly selected to create three experimental variants: V1 - Control, fish fed with a normal diet, V2 - fish fed supplemented with 1% fructose, V3 - fish fed supplemented with 2% fructose, respectively.

Phase II. After 90 days of feeding, 12 from each experimental variant were selected, and the experimental variants were as follows: V1 - Control, fish fed with a normal diet, V2- fish fed supplemented with 1% krill oil, V3 - fish fed supplemented with 2% krill oil (Figure 1). This phase lasted for 40- days. During this trial, fish were fed two times a day at 08:00 a.m. and 4:00 p.m. at a rate of 5% of their body weight, with a commercial diet (37% protein, 12% fat, 4% fiber, 6% ash).

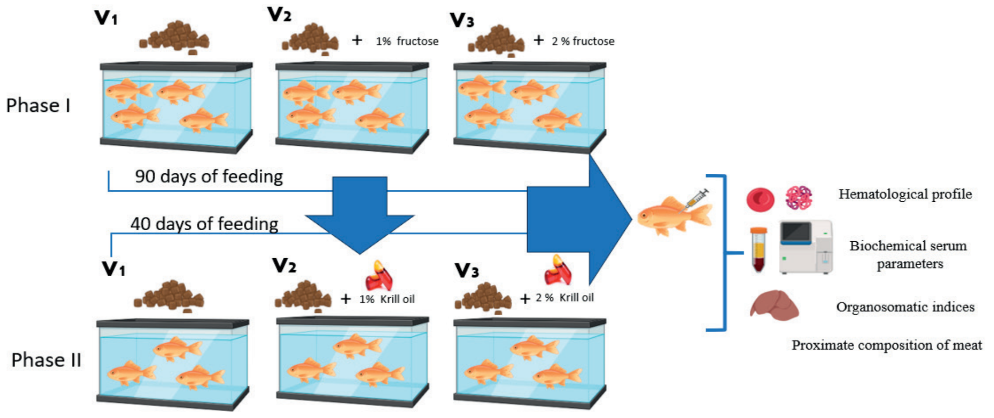

Figure 1. Experimental design (original - Created with BioRender.com)

The experimental activity took place at the Romanian Center for Modelling Recirculating Aquaculture Systems (www.moras.ugal.ro) from the "Dunărea de Jos" University of Galați, Romania. For the experimental activity, six aquariums were used, with a volume of 40 L each. During the experimental period, the water quality parameters such as dissolved oxygen, temperature, and pH were measured daily with the YSI multi-parameter (YSI Pro1020). Weekly we quantify the concentration of nitrogen compounds with the help of the Spectroquant Nova 400 photometer compatible with Merck kits. The average and standard deviation (±SD) of water quality parameters monitored throughout the research were: water temperature was 23.40 ± 0.61°C, pH 7.4, DO was 6.6 ± 0.03 mg L^{-1}, nitrate was 4.9 ± 0.6 mg L^{-1}, nitrites 0.01±0.02 mg L^{-1} and ammonia 0.05 ± 0.01 mg L^{-1}.

Hematological and biochemical parameters. After 90 days of fructose administration (phase I) and after 40 days of feeding with a supplemented diet with krill oil (Phase II) blood samples were collected. Before blood collection fish were gently caught and placed in phenoxyethanol (0.7 mL/L) until deep anesthesia. Blood samples were obtained from five fish in each aquarium through caudal venous puncture, utilizing lithium heparin as an anticoagulant. Subsequent blood analysis employed routine methods commonly applied in fish hematology (Svobodova et al., 1991)

The red blood cells (RBC × 10^6/µL) were counted using glass blood diluting pipette Vulpian diluting solution and a Neubauer hemocytometer. Hemoglobin concentration (Hb, g/dL) was determined utilizing the cyanmethemoglobin method with Drabkin's reagent. Hematocrit (PCV, %) was assessed through the microhematocrit method, involving the centrifugation of blood at 12,000 rpm for 5 minutes. Hematological indices, including mean corpuscular volume (MCV, fL), mean corpuscular hemoglobin (MCH, pg), and mean corpuscular hemoglobin concentration (MCHC, g/dL), were calculated based on the values of PCV, Hb, and RBC, following the methodology outlined by Blaxhall & Daisley in 1973.

The VetTest® Chemistry Analyzer and IDEXX VetTest kits (IDEXX Laboratories, Inc., Westbrook, ME, USA) were employed for the biochemical analysis. Plasma required for the determination of biochemical parameters such as glucose (GLU, mg/dL), alanine aminotransferase (ALT, U/L), aspartate aminotransferase (AST, U/L), and triglycerides (TG, mg/dL), was obtained by centrifuging blood at 3500 rpm for 10 minutes.

Organosomatic Indices. After the end of phase I and II, 6 fish from each experimental variant were sacrificed and the weight of the liver, gonads, and viscera were assessed, for the calculation of hepatosomatic and viscerosomatic indices:

- Hepatosomatic index (HSI, %) = [liver weight (g)/body weight (g)] × 100;
- Viscerosomatic index (VSI, %) = [viscera weight (g)/body weight (g)] × 100;

Proximate Composition Analysis.

The proximate composition analyses were conducted by the established procedures outlined by the Association of Official Analytical Chemists (AOAC, 1997). In this context, 6 fish from each experimental variant were used for the determination of water, lipid, ash, and protein content. To determine dry matter, samples were subjected to drying until a constant weight was achieved at 105°C for 24 hours in a convection oven (Jeiotech, Jeio Tech Co., Inc, Korea). After moisture content determination, the dry samples were finely ground for the analysis of lipids, protein, and ash. Lipid content (%) was assessed through the Soxhlet extraction method, utilizing petroleum ether as the solvent (Gerhardt GmbH & Co. KG, Germany). The ash content (%) was determined by employing a muffle furnace (Nabertherm, Applied Scientific Instruments Co., Ltd. Thailand) at 525±25°C for 8 hours. The crude protein content (%) was calculated by converting the nitrogen content, quantified by Dumas's method through the combustion of dry samples at 1100°C (Primacs SNC 100, Skalar Analytical B.V., The Netherlands), using the common conversion factor of N×6.25.

The fish experiments were conducted with ethical approval obtained from the Ethics Committee of the "Dunărea de Jos" University of Galați, Romania.

Statistical analysis of the data was performed using SPSS software (version 26.). Data are expressed as mean ± SD. One-way ANOVA was employed to assess statistical differences between the experimental variants. The Duncan multiple range test further evaluated significant differences among the groups. The chosen level of significance was set at $p < 0.05$.

RESULTS AND DISCUSIONS

At the end of the two experimental stages were analyzed the fish hematological parameters such as hemoglobin (Hb), red blood cell count (RBC), hematocrit (PCV), and the erythrocytes indices (MCV, MCH, MCHC) (Table 1).

Table 1. Hematological parameters of goldfish

	Hematological parameters	V1	V2	V3
Phase I	Hb (g/dL)	9.77± 0.88^{a}	9.33± 0.75^{a}	9.51± 1.03^{a}
	PVC (%)	32.04± 1.63^{a}	31.67± 3.04^{a}	32.42± 5.11^{a}
	RBCc ($\times10^6/\mu L$)	1.49± 0.18^{a}	1.52± 0.26^{a}	1.51± 0.17^{a}
	MCV (fL)	215.03± 12.44^{a}	208.35± 19.08^{a}	214.70± 21.52^{a}
	MCH (pg)	65.57± 9.21^{a}	61.38± 12.15^{a}	62.98± 20.31
	MCHC (g/dL)	30.49± 5.97^{a}	29.46± 8.65^{a}	29,33± 6.02
Phase II	Hb (g/dL)	10.21± 1.86^{a}	9.26± 1.10^{a}	9.47± 1.21^{a}
	PVC (%)	31.79± 2.41^{a}	30.19± 5.35^{a}	32.8± 5.66^{a}
	RBCc ($\times10^6/\mu L$)	1.56± 0.19^{a}	1.50± 0.34^{a}	1.66± 0.09^{a}
	MCV (fL)	192.01± 16.41^{a}	208.51± 78.98^{a}	197.37± 33.91^{a}
	MCH (pg)	61.67± 11.49^{a}	64.40± 18.14^{a}	57.11± 8.78^{a}
	MCHC (g/dL)	32.26± 6.68^{a}	33.49± 12.34^{a}	29.42± 5.08^{a}

Data are expressed as the Mean ± SD. Different letters indicate significant differences between experimental variants (ANOVA, $p < 0.05$).

Regarding the influence of different concentrations of fructose, we noticed that there are no statistically significant differences ($p > 0.05$) in the main hematological parameters (Hb, PVC, RBC). At the end of the feeding krill oil experiment, although no significant differences ($p > 0.05$) were detected, the data showed a slight increase in values of RBC in the V3 variant. Haemoglobin content (Hb), hematocrit (PVC), mean corpuscular volume (MCV), mean corpuscular hemoglobin content (MCH), and mean corpuscular hemoglobin concentration (MCHC) were not significant ($p > 0.05$) affected by dietary krill oil. At the end of the trial and after the krill oil supplementation, the metabolic profile of the fish was analyzed, plasma biochemical parameters values (mean ± SD) (AST, ALT - aspartate and alanine amino-transferase, TRIG - triglycerides, and GLU - glucose) are presented in Table 2.

Table 2. Serum metabolic profile parameters of goldfish

Exp. design	Biochem Param.	V1	V2	V3
Phase I	AST (U/L)	61.33± 2.34^{a}	59.50± 3.65^{a}	71.67± 5.07^{b}
	ALT (U/L)	417.00± 14.08^{b}	341.50± 16.05^{a}	532.67± 17.64^{c}
	GLU (mg/dL)	74.67± 3.49^{a}	77.00± 4.06^{a}	78.33± 4.21^{a}
	TRIG (mg/dL)	158.00± 5.22^{a}	184.50± 4.88^{b}	205.00± 7.21^{b}
Phase II	AST (U/L)	56.00± 3.16^{b}	49.00± 3,31^{a*}	46.00± 2.40^{a*}
	ALT (U/L)	398.00± 15.44^{b}	315.00± 21.17^{a*}	423.50± 17.23^{b*}
	GLU (mg/dL)	80.50± 2.78^{a}	81.00± 2.44^{a}	81.50± 3.07^{a}
	TRIG (mg/dL)	155.5± 7.02^{a}	180.5± 8.87^{b}	174.00± 8.05^{b*}

Values are Mean±S.D., Values with different superscripts in a column differ significantly (ANOVA, $p < 0.05$). Values with different symbols * in a row differ significantly after krill oil ($p < 0.05$).

Before the Krill oil supplementation, fish fed with 1% and 2% fructose registered significant changes in some biochemical parameters compared with groups fed with a normal diet. Fructose 1% did not induce significant changes in AST, ALT, and GLU ($p > 0.05$), except TRIG, which had higher values, bat AST, ALT, and TRIG significant increase in fish feed with fructose 2% ($p < 0.05$). After Krill oil supplementation, significant decreases were observed in the ALT, AST, and TRIG values from V3 ($p < 0.05$), while in version V2 the changes in biochemical parameters were statistically insignificant ($p > 0.05$).The effects of different concentrations of dietary krill oil on the chemical composition of the whole body of *Carassius auratus* var. *calico* are shown in Figures 2, 3, and 4. No significant differences were shown in whole-body moisture, protein, lipid, and ash among the dietary treatments ($p>0.05$).

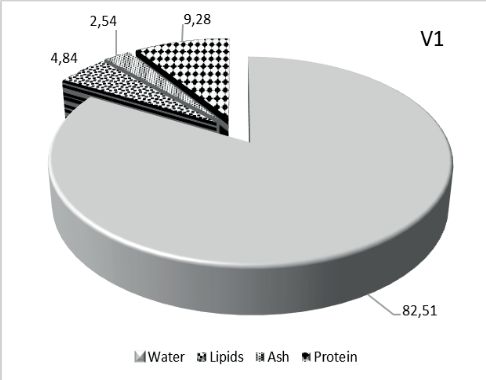

Figure 2. The biochemical composition of fish at the end of phase II

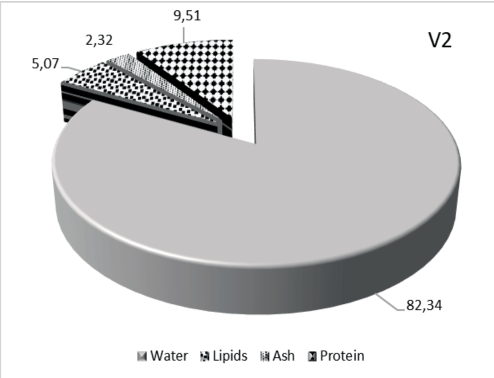

Figure 3. The biochemical composition of fish at the end of phase II

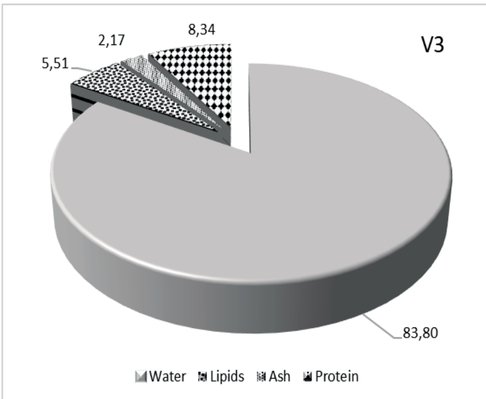

Figure 4. The biochemical composition of fish at the end of phase II

Table 3 presents the values of organosomatic indices at the end of the two experimental stages. In the case of our experiment, ANOVA statistical analysis revealed no significant differences (P>0.05) for the values of HIS and VSI indices.

Table 3. The viscerosomatic and hepatosomatic index of goldfish

Period	Experimental variants	HSI	VSI
Phase I	V1 Control	3.33± 0.07[a]	9.66± 1.68
	V2 1% fructose	3.97± 0.03	10.37± 0. 85[a]
	V3 2% fructose	4.20± 0.07	10.99± 1.13
Phase II	V1 Control	3.80± 1.13[a]	16.72± 3.81[a]
	V2 1% Krill	3.67± 1.49	13.09± 5.23[b]
	V3 2% krill	3.97± 1.06	13.29± 5.10[b]

Data are expressed as the mean ± SD. Different letters indicate significant differences between experimental variants (ANOVA, $p < 0.05$).

Carbohydrates are an important ingredient in fish diet, but the dosage is critical. Fructose is known as a pro-inflammation metabolite in mammals (Jones et al., 2021). The excessive intake of fructose is harmful to health, especially for organisms with chronic diseases (Velickovic et al., 2019). High fructose (10.5 g/kg) significantly increases uric acid and proinflammatory cytokines in serum (Wang et al., 2020). On the other hand, some results indicated the beneficial effect of fructose as a metabolite in promoting *C. carassius* survival and also report that fructose mainly enhances the expression of lysozyme and complement, which enables the bacteria to be reduced more efficiently (Cao et al., 2022).

Several studies examining the influence of diet on various hematological parameters have determined that alterations in these parameters occur in response to changes in dietary composition. This underscores a correlation between food intake and hematological variations in the investigated fish species. (Abdulrahman et al., 2019; Satheeshkumar et al., 2012; Hoseinifar et al., 2011; Abdel et al., 2006).

The findings from our current study indicate that a diet containing fructose had no discernible impact on hematological parameters. Similarly, after the experiment, supplementation with krill oil also demonstrated minimal effects, with only a slight increase observed in the number of erythrocytes. The slight increase in erythrocyte count following dietary supplementation with krill oil may be due to krill's iron intake (John et al., 1983), which is known to be an essential component of hemoglobin. Krill also contains vitamins and minerals (B12, B9) (Xie et al., 2019) essential for the formation of red blood cells.

Analyzing blood parameters provides insights into the physiological condition of fish and can be indicative of various health-related factors. The interest in such studies has increased in recent years due to the importance of understanding the baseline values of blood parameters and how they can be influenced by a variety of factors (Ahmed et al., 2020). Nutrition plays a crucial role in the overall health of fish, and the composition of their diet can affect blood parameters (Esmaeili, 2021; Kenari et al., 2011). For example, the quality and quantity of nutrients in the fish's diet can influence parameters like hematocrit, hemoglobin levels, and various metabolic indicators (Azaza et al., 2020).

Alterations in the composition of blood serum components can be utilized to detect specific functional disorders in the organs and assess the overall health status of fish (Li et al., 2023). In our study, we evaluated the variation of some biochemical parameters (AST, ALT, GLU, TRIG) to highlight the beneficial role of krill oil in the case of administration of a high-carbohydrate diet. After ingesting a high-carbohydrate diet (1 % and 2% fructose), there was no apparent effect on plasma glucose levels, indicating that goldfish are capable of regulating glycemia effectively. Other researchers have found comparable results: stable plasma glucose levels may result from the liver and muscle's increased lipogenesis potential and the absence of changes in gluconeogenesis (Li et al., 2019).

In omnivorous fish, as in other animals, AST (Aspartate Aminotransferase) and ALT (Alanine Aminotransferase) values can serve as indicators of liver health (Coz-Rakovac et al., 2008). These enzymes are predominantly found in the liver, and elevated levels may suggest liver dysfunction or damage (Ali et al., 2017).

The notable increase in ALT and AST levels in the V3 - fish feed with 2% fructose compared to the V1 variant, may indicate potential stress in the hepatopancreas of goldfish under the present study. However, lower ALT and AST values observed in V2 groups (fed with 1% fructose) may be associated with the anti-inflammatory effect of low fructose concentrations reported also for *C. carassius* by other authors (Cao et al., 2022). Therefore, in our study, 2% of fructose imposed a higher pressure on the hepatopancrease activity.

After 40 days of krill oil 2% supplementation there was an observed improvement in hepatopancrease function, as evidenced by a significant decrease in liver enzyme values (AST, ALT).

Fructose is known to stimulate lipogenesis, a process where the liver converts excess carbohydrates into triglycerides (Shikata et al., 1994); this can result in increased production and release of triglycerides into the bloodstream, an aspect also observed in our study at the end of stage I of growth. For goldfish, after the feeding trial, at the end of stage II, a krill oil 2% supplemented diet induced a significant decrease in TRIG concentration, suggesting that KO alleviated the negative impact of fructose on the metabolism of lipoproteins.

Gaining insights into specific quantitative aspects of fish is essential for delving into fundamental biological concepts, such as the examination of viscerosomatic and hepato-somatic indices. This is due to the significance of measuring and analyzing these indices in evaluating the nutritional value of administrated food (Ighwela et al., 2014). The hepatosomatic index serves as an indirect metric for assessing the nutritional status of fish by measuring glycogen and carbohydrate levels (Tavares-Dias et al., 2000).

Although, at the end of Phase I, no significant differences were observed between hepato-somatic and viscerosomatic indices, a slight increase of HSI (hepatosomatic index) and VSI (viscerosomatic index) can be noticed in the V3 variant (2% fructose). Typically, the increase in HSI observed alongside the rise in dietary carbohydrate levels could be associated with an increase in glycogen accumulation within the liver of fish fed to high-carbohydrate diets (Moreira et al., 2008; Li et al., 2015). The same trend of non-significant increase is observed at the end of Phase II for the HSI, while the VSI exhibited a significantly higher value in the variant where the fish diet was not supplemented with krill oil. Krill oil, rich in omega-3 fatty acids and antioxidants, may decrease viscerosomatic indices in fish through its anti-inflammatory effects (Ku et al., 2023), regulation of lipid metabolism, antioxidant protection, and maintenance of cellular membrane integrity (Sun et al. 2017).

CONCLUSIONS

The present study was conducted to investigate the amelioration of hepatopancreas impairment induced by high fructose dietary doses following dietary supplementation with krill oil for *Carassius auratus* reared in different strategy-feeding. Our results showed that daily krill oil supplementation in the *C. auratus* prevented fructose-induced hypertriglyceridemia. Therefore, monitoring the blood biochemical parameters can help in optimizing feeding practices to ensure the welfare and productivity of the fish. Research in this area is ongoing, and studies aim to optimize the incorporation of krill oil into aquafeeds, taking into account economic feasibility and environmental impact.

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NEW DATA FOR HELMINTH FAUNA OF *Rana temporaria* (Linnaeus, 1758) IN THE REPUBLIC OF MOLDOVA

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Abstract

The paper presents data on the identification for the first time in Moldova of the helminth fauna structure of Rana temporaria and the determination of its role as bioindicators and as vectors for parasitic agents specific to animals. As result of helminthological investigations during 2014-2023 years, 19 helminths species was established. The predominant group are trematodes with 57.8% of cases, nematodes with 31.6% of cases, acanthocephala and monogeneans with 5.3% of cases each. According to the assessment of the main helminthological indices, it was established that the species is infested with helminths in 96.3% of cases. When evaluating the data and the composition of helminth species, it was found that adult forms of helminths are predominant over larval forms, so that, when the host species is infected with trematodes, 9.1% are metacercariae, and 90.9% are the adult forms, when the host species is infected with nematodes 33.3% are the larval stages, and 66.7% are the mature forms, when infesting the host species with acanthocephals and monogeneans, it was established that the helminth species are an adult form, each constituting 5.3%.

Key words*: bioindicators, Moldova, parasitic agents, Rana temporaria, vectors*

INTRODUCTION

Rana temporaria (Common frog) is a species of ecaudate amphibians from Ranidae family with a wide distribution area, found throughout Europe, except for Southern Italy and the Southern Balkans. In the Republic of Moldova, the common frog is characterized by a limited distribution, inhabiting only the northern part of the country, up to the border of Ungheni, Călărași (Sipoteni, Bucovăț), Criuleni and Maiac localities (Cozari et al., 2007; Arnold & Burton 1986, Bannikov et al., 1977, Cozari & Gherasim, 2021).

Currently this species is included in the Red Book of the Republic of Moldova (2015) with the status of vulnerable species (VU), but according to IUCN category 2018-1 it has the status of LC (Least Concern).

The reduction of the population of the *Rana temporaria* species represents its response to the direct action of anthropogenic transformation of the environment, which is currently quite advanced, and contributes directly to the destruction of specio-specific habitats, the actual destruction of specimens, as well as the degradation of biodiversity in general (Cozari & Gherasim, 2021).

Another factor that directly contributes to the reduction of the *Rana temporaria* population is represented by the specific biocenotic relationships in the ecosystems populated by it. As a result of these interactions, certain specific relationships are formed in the parasite-host system, which also includes a cryptic ensemble of parasitic agents, and the *Rana temporaria* species serves as a host for at least one developmental stage of the parasitic agents.

Although the parasitic fauna of the *Rana temporaria* species is a constitutive part of both aquatic and terrestrial biotopes, also representing as actual bioindicators of its specific ecosystems, the parasitic agents have a negative impact on the perpetuation of amphibians as living organisms, thus producing certain dysfunctions of the organ systems of amphibians, and respectively to the reduction of the *Rana temporaria* population.

Internationally, helminthological research on this species of ecaudate amphibians from the Ranidae family has been carried out and described by more authors (Griffin, 1989;

Chikhlyaev & Ruchin, 2014; Herczeg et al., 2021; Burakova & Malkova, 2021).

In the Republic of Moldova, the amphibian species *R. temporaria* has been researched from a biological, taxonomic, ecological, genetic, evolutionary, etc. point of view (Cozari & Gherasim, 2021), but complex helminthological research was carried out for the first time, and the results are presented in this scientific paper.

MATERIALS AND METHODS

The study area includes the Center area (Călărași, Străseni, Codrii Centrali Hâncești, Telenești, Sângerei Districts) of the Republic of Moldova. In the context of the principles of helminthological research of an organism (species) it is extremely important to apply certain appropriate and effective concepts of species-host identification. In this context, the biological and morphological concepts are of major importance, although each of them, taken separately, also presents certain methodological difficulties.

The amphibian species *Rana temporaria* was identified using of morphometric parameters (Arnold & Burton 1986, Bannikov et al., 1977; Kuzmin, 2012).

The helminthological analysis of biological samples was performed according to the standard method proposed by K.I. Skriabin, which involves the examination of all the internal organs of the animal (Skriabin, 1928). 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 (Gashev et al., 2006; Petrochenko, 1956; Erhan & Gherasim, 2022; Sergiev 2001; Sudarikov, et al., 2002; Shchepina et al., 2006).

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

Rana temporaria Linnaeus, 1758 species is a species of ecaudate amphibians of the Ranidae family, the group of brown frogs, which emerges very early from the hibernation phase, when the environmental temperature is not ecologically optimal for a wide range of invertebrate animals, which are obligate intermediate hosts (insects, artropods, crustaceans, etc.), for its specific parasitic fauna.

As a result of the helminthological investigations carried out on the common frog during its entire annual and life cycle, the presence of 19 species of helminths was established, which from a taxonomic point of view are included in 3 phylums (Platyhelminthes, Nematoda, Acanthocephala), four classes (Trematoda, Monogenea, Secernentea, Palaeacanthocephala), eight orders (Plagiorchiida, Echinostomida, Ascaridida, Strongylida, Spirurida, Rhabditida, Echinorhynchida, Polystomatida), 15 families (Omphalometridae, Cepahlogonimidae, Gorgoderidae, Lecithodendriidae, Pleurogenidae, Diplodiscidae, Plagiorchiidae, Diplostomatidae, Macroderoididae, Cosmocercidae, Molineidae, Spirocercidae, Rhabdiasidae, Echinorhynchidae, Polystomatidae) and 18 genres (*Opisthioglyphe, Cephalogonimus, Gorgodera, Gorgoderina, Pleurogenes, Pleurogenoides, Prosotocus, Diplodiscus, Haplometra, Plagiorchis, Tylodelphys, Cosmocerca, Oswalcocruzia, Ascarops, Agamospirura, Rhabdias, Acanthocephalus, Polystoma*).

Therefore, in the *Rana temporaria* species from the 19 detected species of helminths, the predominant group are trematodes (n=11) which constitute 57.9% of cases, nematodes with 31.6% of cases (n = 6) and acanthocephals (n = 1) and with monogeneans (n = 1) with 5.3% of cases each (Figure 1).

To evaluating of the data and the structure of helminth species, it was found that the adult forms of helminths are predominant over the larval forms, so that when the host species are infected with trematodes, 9.1% are metacercariae (*Tylodelphys excavata*), and 90.9% are the mature forms (*Opisthioglyphe ranae, Cephalogonimus retusus, Gorgodera*

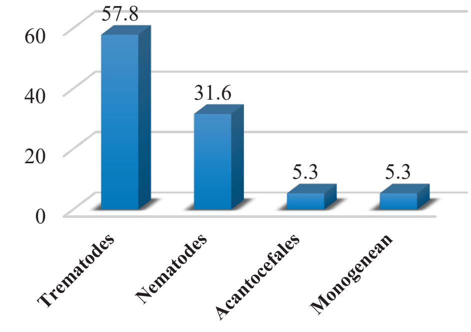

Figure 1. Indices of extensivity of the taxonomic classes of helminths detected in the *Rana temporaria* species

varsoviensis, Gorgoderina vitelliloba, Pleurogenes claviger, Pleurogenoides medians, Prosotocus confusus, Diplodiscus sbclavatus, Haplometra cylindracea, Plagiorchis elegans), when the host species is infected with nematodes 33.3% are the larval stages and 66.7% are the mature forms, to the infestation of the host species with acanthocephals and monogenean it was established that the helminth species are an adult form, each constituting 5.3% (Figure 2).

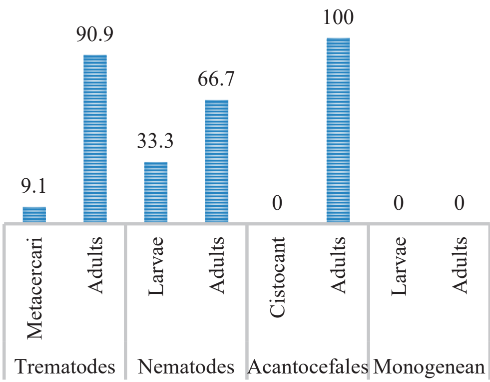

Figure 2. Helminthological indices of the *Rana temporaria* species depending on ontogeny parasitic agents

Therefore, during the helminthological investigation of *Rana temporaria* species it was established that this is a host species that is predominantly infested with invasive elements of adult form.

Therefore, according to the evaluation of the structure of the helminthic fauna in the host species *Rana temporaria* depending on the ontogenetic phases of the detected helminths, it was found that this host for 15.8% of the total number of specific helminth species constitutes a truthful vector.

At the same time, the degree of vectorization of parasitic agents by the ecaudate amphibian species *Rana temporaria* from the Raniade family to wild, domestic, companion animals and humans is explained not only thanks to the trophic relations in the ecosystem (prey-predator), but also the possibility of its simultaneous infestation with several species of parasitic agents.

Thus, according to our data for the *Rana temporaria* species, it was established that in 7.7% of the cases the specimens were infested with a single species of helminths, 46.1% of the cases - with 2 species of helminths, 23.1% of the cases - with 3 species of helminths, 15.4% of cases - with 4 species of helminths and 7.7% of cases - with 5 species of helminthes (Figure 3).

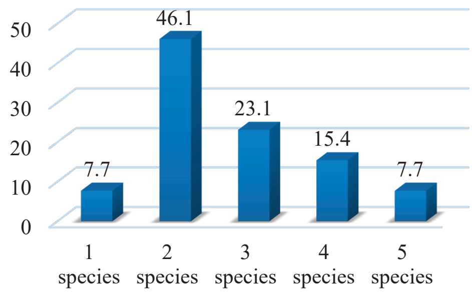

Figure 3. Degree of co-infection of the *Rana temporaria* species

It is known that amphibians have a big important role in the functioning of ecosystems as consumers and in regulating the population of invertebrates in an ecosystem.

According to our obtained data, the role of the ecaudate amphibian species *Rana temporaria* was determined as the definitive host for 84.2% of cases of helminth species (*Cosmocerca ornata, Oswaldocruzia filiformis, Oswaldocruzia duboisi, Rhabdias bufonis, Polystoma integerrimum, Diplodiscus subclavatus, Acanthocephalus ranae, Gorgodera varsoviensis, Gorgoderina viteliloba, Pleurogenes claviger,* *Pleurogenoides medians, Prosotocus confusus, Cephalogonimus retusus, Haplometra cylindracea, Opisthioglyphe ranae, Plagiorchis elegans*), intermediate for 10.5% of cases (*Agamospirura sp., Tylodelphis excavata*) and as a paratenic host for 5.3% of cases (*Ascarops strongylina*) for helminth species common to fish, reptiles, birds, mammals and humans (Figure 4).

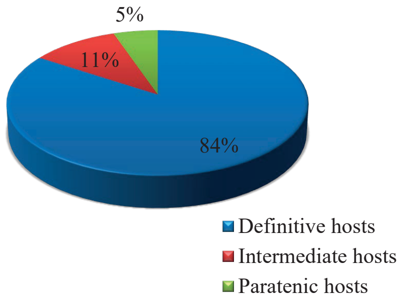

Figure 4. The role of *Rana temporaria* species as a host of the parasitic agents

If in the life cycle of helminths specific to amphibians or other groups of animals (reptiles, birds, mammals) there is an intermediate host (mollusks, insects, amphibians), then it does not participate at all in the search for the next host (definitive host). Of course, the probability of infection increases if the parasite hits the right host and exerts some influence on that host, making it more attractive to a next potential host. According to the ethological analysis of amphibians, it was observed that those amphibian specimens that were infested with metacercariae had a delayed response to the action of biological factors (predators) in the environment. But the insight of the parasite into the next host depends entirely on its nutritional biology, which the parasite cannot influence in any way. If, however, the parasite is found in a suitable (specific) intermediate host, then, due to the nutritional relationships existing in the given habitat, it is limited to a certain set of possibilities and consequences. Thus, the completion of the entire life cycle of the parasite is predetermined and, thanks to this fact, the probability of meeting the appropriate definitive host increases impressively. The parasite does not choose its next host and does not find it as such, but must be able to recognize it as a potential host. If a parasite found in an unsuitable host, it either leaves it immediately or lives in it for a limited period of time and then dies.

The role of amphibians as paratenic hosts, according to our data, represents another strategy of parasite adaptation to the living environment. This occurs when parasite larvae or intermediate hosts infected with it are consumed not by obligate hosts of this parasite, but by amphibians, representing a nutritional necessity of animals. Unlike definitive or intermediate hosts, which are obligatory for the life cycle of a particular parasite, these accidental hosts are facultative, in which, parasites do not develop but simply live for a certain period of time without losing their infectivity to the next host (Gherasim & Erhan, 2024).

Facultative hosts can never replace intermediate hosts, but they can intervene in the life cycle of the parasite at almost any of its stages. The importance of amphibians as facultative hosts in the transmission of parasites to a suitable host is determined by how close are the connection of the food spectrum between successive hosts of the parasite (invertebrates - amphibians - vertebrates) (Gherasim & Erhan, 2024).

For many parasites, the probability of encountering a new host may be negligible if the host population is small or highly dispersed. In such cases, the infection is possible only when hosts form short-term aggregations, such as during the breeding season in amphibians. At the same time, it is absolutely necessary for some parasites to find and infect specimens (hosts) belonging to a new generation of the host. Therefore, the life cycle of such parasites is often synchronized with that of the hosts, with infective larvae developing and being released just as the host specimens form groups or produce offspring. Such synchronization is achieved by the same response of both host and parasite to a physical factor, either because the parasite's reproduction depends by the reproduction of the host or it is directly regulated of this.

Polystoma integerrimum species is a parasite of amphibians, which behaves similarly. Its sexual organs mature just as the amphibians get in the water to breed, and this parasite only lays eggs during the period (which lasts about a week) when the amphibians are in the water and forming couples (specific only to amphibians that use water pools only for breeding, except for the *Pelophylax esculenta* amphibian complex: *Pelophylax ridibundus, Pelophylax lessonae, Pelophylax esculentus*). Necessary time for eggs to develop into larvae is equal to the necessary time for amphibian eggs to develop into tadpoles and the parasite to reach the stage where they develop in the gills. Thus, the oncomiracidia capable of invasion and the hosts they presumably infect arrive at the same place at the same time.

In this sense, the amphibians being intermediate or paratenic hosts for a series of larval stages of helminths (eggs, cercariae, metacercariae, cystocanth, larvae, etc.), they fulfill a particularly important role in the epidemiology and epizootology of helminthes infestations of wild animals, domestic, companion and human, and their impact on the definitive hosts (vertebrates) is little studied, or even completely missing.

Although scientific research is characterized by an impossible to control scale, still, until now there are species of helminths whose biological cycle is still unknown, and their hosts could be animals of economic importance (cattle, goats, horses, sheep etc.) as well as human. Once helminthological research is carried out in amphibians, their role in the vectorization of helminthiasis in wild animals, pets, farm animals and humans can be identified.

When evaluating the degree of vectorization of parasitic agents by the host species *Rana temporaria* in the areas populated by it, it was determined that of the total of helminth species detected, 2 species (*G. varsoviensis, T. excavata*) are common to fish, which constitutes 10.5%, 2 species are common to reptiles (*P. elegans, A. strongylina*) which represents 10.5% of cases, 3 species of helminths detected in the host *Rana temporaria* (*P. elegans, T. excavata, A. strongylina*) are specific to birds and constitute 15.8% of cases, 3 species are common to mammals (*P. elegans, A. strongylina, Agamospirura* sp.) representing 15.8% of cases and one species can also be found in humans (*P. elegans*) which represents 5.3% of cases (Figure 5).

Usually, the parasites tend to have a complex life cycles, and it is unlikely that only one host is required in which to grow and therefore reproduce and form new generations. If all parasitic agents were to survive, then their abundance would far exceed that of their hosts, thus creating a potential threat to the existence of the host population.

Over-infestation of the host is always a potential danger to the parasite. Any parasite population, which overuses the available resources, has a chance to survive, and it is the host that provides the parasite with essential resources for survival, such as food and favorable conditions for growth, development and formation of new generations.

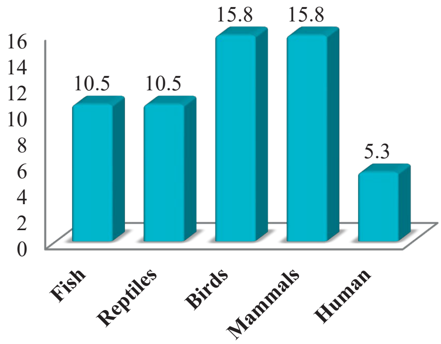

Figure 5. The degree of vectorization of parasitic agents by the amphibian species *Rana temporaria*

At the basis of the perpetuation of any species is its certain ethological criteria regarding leaving the parental habitats, or a population by populating new areas, but avoiding its overpopulation, which usually leads to mortality (Gherasim & Erhan, 2024).

For parasites, the dispersal plays a particularly important role, as over infestation of hosts is a permanent threat to them. Therefore, both adult parasites and their larval stages must possess the ability to leave the host organism and at the same time find other new hosts. Sometimes the new host may belong to the same species, but the need for the parasite to be in another host organism of another generation is constant.

In both cases, the parasite spends a certain stage of its life cycle outside the host organism, in the environment. Dispersal often occurs at this stage, but since the probability of finding a new host is generally extremely low and parasite mortality during the dispersal period can be very high. To compensate for this mortality, the parasites must have a very high fecundity, and this is also facilitated by their asexual or parthenogenetic reproduction.

The intermediate hosts, thanks to their own mobility, contribute to the spread of the parasite in the environment and, thanks to the ecological relationship's that exist between them and the definitive hosts, facilitate its penetration into the latter's body. Parasite behavior in an intermediate host or the behavior of the host itself may also increase the probability of finding a definitive host.

Therefore, the monitoring of the parasite fauna in amphibians, in various biotopes, depending on the intrinsic and extrinsic factors, it has a bioecological, medical and veterinary importance in preventing the transmission of the parasitic agents to humans and animals involved in the biological cycles of parasites with a zoonotic and epizootic role.

Therefore, the helminthological research in amphibians allows us to conclude that the helminthic fauna of these host organisms, with the amphibiotic way of life, is of particular importance not only theoretically, but also practically, actively participating in the formation and maintenance of foci of parasitic agents common to fish, birds, mammals and human.

CONCLUSIONS

For the first time in the Republic of Moldova, helminthological investigations were carried out on the species of ecaudate amphibians *Rana temporaria* Linnaeus, 1758.

As a result of the helminthological research carried out, the presence of 19 species of helminths was established, which from a taxonomic point of view are included in three phylum (Platyhelminthes, Nematoda, Acanthocephala), four classes (Trematoda, Monogenea, Secernentea, Palaeacanthocephala), eight orders (Plagiorchiida, Echinostomida, Ascaridida, Strongylida, Spirurida, Rhabditida, Echinorhynchida, Polystomatida), fifteen families (Omphalometridae, Cepahlogonimidae, Gorgoderidae, Lecithodendriidae, Pleurogenidae, Diplodiscidae, Plagiorchiidae, Diplostomatidae, Macroderoididae, Cosmocercidae, Molineidae, Spirocercidae, Rhabdiasidae, Echinorhynchidae, Polystomatidae) and eighteen genres (*Opisthioglyphe, Cephalogonimus, Gorgodera, Gorgoderina, Pleurogenes, Pleurogenoides, Prosotocus, Diplodiscus, Haplometra, Plagiorchis, Tylodelphys, Cosmocerca, Oswalcocruzia, Ascarops, Agamospirura, Rhabdias, Acanthocephalus, Polystoma*).

The structure of the helminthic fauna was studied depending on the direct influence of ecological factors during the entire annual and life cycle of *Rana temporaria* Linnaeus, 1758, species hitch allowed the appreciation of the degree of knowledge of the period, stations and habitats where it is possible to vectorize parasitic agents to the definitive hosts of detected parasitic agents.

It was found that the host species *Rana temporaria* from the area of the Republic of Moldova plays an important role in the formation and maintenance of foci of parasitic agents common to fish in 10.5% of cases, reptiles - 10.5% of cases, birds - 15.8% of cases, mammals - 15.8% of cases and humans in 5.3% of cases.

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HELMINTH FAUNA OF *Rana dalmatina* (Bonaparte, 1840) IN THE REPUBLIC OF MOLDOVA

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Abstract

The paper presents data on the identification for the first time in Moldova of the helminth fauna structure of Rana dalmatina Bonaparte, 1840, and the determination of its role as bioindicators and as vectors for parasitic agents specific to animals. As result of helminthological investigations, 18 helminths species was established. The predominant group are trematodes with 61.1% of cases, nematodes with 27.7% of cases, acanthocephalan and monogeneans with 5.6% of cases each. According to the assessment of the main helminthological indices, it was established that the species is infested with helminths in 83.3% of cases. When evaluating the data and the composition of helminth species, it was found that adult forms of helminths are predominant over larval forms, so that, when the host species is infected with trematodes, 27.3% are metacercarias, and 72.7% are the adult forms, when the host species is infected with nematodes 20.0% are the larval stages, and 80.0% are the mature forms, when infesting the host species with acanthocephalans and monogeneans, it was established that the helminth species is an adult form, each constituting 5.6%.

Key words*: Bioindicators, Moldova, parasitic agents, Rana dalmatina, vectors.*

INTRODUCTION

One of the indispensable components of the fauna are amphibians, which following the degradation of natural ecosystems, represent a low diversity of species. In the last decades, in the area of Central Forests on the territory of the Republic of Moldova, certain local populations of amphibians have disappeared, or some species such as *Bombina bombina*, *Hyla arborea*, *Triturus cristatus*, *Triturus vulgaris* and *Rana dalmatina* they considerably reduced their numerical effective (Cozari & Gherasim, 2021; Plop, 2011). The multi-year dynamics of the population of amphibian species, especially the *Rana dalmatina* Bonaparte, 1840 species, allowed us to note that this situation was recorded on the entire territory of our country, but as well as on a regional and continental level.

The direct action of the climate changes, global warming, fluctuations in the seasonal thermal regime not specific to a certain area, environmental pollution and the anthropic factor directly contribute to the formation of biocenotic relationships in the ecosystem and its functionality.

As a result of these threats, the *Rana dalmatina* Bonaparte, 1840 species was included in the list of protected species in Annex no II of the Berne Convention, the Habitats Directive of the Convention on the Conservation of Wild Life and Natural Habitats in Europe. Currently, the IUCN has assigned the *Rana dalmatina* species the status of Least Concern (LC) species. In the Red Book of the Republic of Moldova (2015) it is characterized as a vulnerable species (VU).

Although, the continuous influence of abiotic and anthropogenic ecological factors reduce the population of the *Rana dalmatina* species on the territory of our country, however, the reduced population of this amphibian species is also determined by the direct influence of biotic ecological factors, which directly contribute to the formation of relationships direct and indirect interspecific (trophic, topical, phoric/vectorization) between the *Rana dalmatina* species and the living organisms in the ecosystems populated by it.

According to the adaptability of *Rana dalmatina* species to the conditions of its environment, but also due to the species-specific ecological relationships existing from an evolutionary point of view, it is possible to form a specific parasitic structure, which certainly has an impact on its population.

In this sense, the amphibian species *Rana dalmatina* through its specific parasitological structure, but also the trophic relations in the ecosystems populated by it, represents a true biological indicator of the ecosystems, but at the same time favors the transmission of its parasitic agents for other groups of vertebrate animals.

According to the helminthological research carried to specimens of the *Pelophylax esculenta complex* (*Pelophylax ridibundus*, *P. lessonae*, *P. esculentus*) on the territory of the Republic of Moldova, it was demonstrated that amphibians have an important role in the formation and maintenance of foci of the parasitic agents specific to domestic, wild, pet and human (Gherasim, 2016; Gherasim, 2023a; Gherasim, 2023b).

On a regional level, in different areas of the world, helminthological research was carried out on various species of amphibians (Düşen et al., 2009; Chikhlyaev et al., 2018), including the *Rana dalmatina* species.

On the territory of the Republic of Moldova, this species is especially distributed in the natural ecosystems of the Central and Northern areas of the country, and helminthological research on this species of ecaudate amphibians was carried for the first time.

Therefore, carrying of helminthological research on the *Rana dalmatina* species is an effective necessity both for the functioning of ecosystems and for the well-being of society.

MATERIALS AND METHODS

In order to determine the structure of the helminthic fauna of *Rana dalmatina* species, complex research was carried out with reference to the phenology of this, its population structure (age structure, spatial structure) as well as helminthological research in the area of distribution of the species - the area of the Central Forests of the Republic of Moldova.

The helminthological analysis of biological samples was performed according to the standard method proposed by K.I. Skriabin, which involves the examination of all the internal organs of the animal (Skriabin, 1928).

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 (Gashev et al., 2006, Kuzmin, 2012, Ryzhikov, 1980).

In order to quantify the characteristics of helminthes contamination, the intensity indexes (II, specimens) were 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.

The helminthological research of the *Rana dalmatina* Bonaparte, 1840 species regarding the determination of the presence of helminthic agents depending on the age structure of the host includes the helminthological evaluation of the different stages of ontogenetic development of the host: egg - larvae - juvenile - adult, which will contribute to the knowledge of the importance of the species in the formation and maintenance of foci of parasitic agents common to wild, domestic animals, pets and human, but also to the development of biological methods to effectively combat parasitic zoonosis.

RESULTS AND DISCUSSIONS

The agile frog is the species of ecaudate amphibians with the earliest breeding period recorded not only on the territory of the Republic of Moldova, but also in Romania and other European countries.

In order to decipher and understand the interaction or relationships in the host-parasite system on the example of the amphibian species *Rana dalmatina*, but also to approach the common effects of the host and parasites on the increased risk of vectorization by specific parasitic agents, the multi-year phenology of this species throughout the country was evaluated.

Thus, according to the multi-year evaluations, in the area of the Central Forests of the Republic of Moldova, the first breeding specimens appeared on the soil surface in the site forestry were registered in the 3rd decade of March - the 1st decade of April (Cozari & Gherasim, 2021), but in Romania, depending on the climatic conditions, it takes place out between February and April months (Fuhn, 1960).

As a result of the data analysis, the active period of the annual life cycle of amphibians was determined and it was established that their phenology is in full accord with the establishment of certain favorable climatic conditions of the temperature and humidity, and all together have an important role in the formation of intra- and interrelationships species-specific ecology of amphibians, with living environments and biological diversity.

In order to determine the structure of the helminthic fauna of the *Rana dalmatina* species, helminthological investigations were carried out, and according to the data obtained, it was established that the agile frog is infected with 18 species of helminthes, which from a taxonomic point of view fits into three phylums (Platyhelminthes, Nematoda, Acanthocephala), four classes (Trematoda, Monogenea, Secernentea, Palaeacanthocephala), eight orders (Plagiorchiida, Echinostomida, Diplostomida, Ascaridida, Strongylida, Rhabditida, Echinorhynchida, Polystomatida), fifteen families (Omphalometridae, Haematoloechidae, Gorgoderidae, Lecithodendriidae, Pleurogenidae, Diplodiscidae, Diplostomatidae, Strigeidae, Macroderoididae, Telorchiidae, Cosmocercidae, Molineidae, Rhabdiasidae, Echinorhynchidae, Polystomatidae) and seventeen genres (*Opisthioglyphe, Haematoloechus, Gorgodera, Gorgoderina, Pleurogenes, Pleurogenoides, Diplodiscus, Strigea, Haplometra, Tylodelphys, Telorchis, Cosmocerca, Oswalcocruzia, Agamospirura, Rhabdias, Acanthocephalus, Polystoma*).

According to the evaluation of the helminthological multiannual data obtained, we will mention that the host species *Rana dalmatina* is characterized by a high degree of infestation, which constitutes 83.3% of the cases, for which the main helminthological indices are very variable, and the extensivity of the invasion (E.I) - oscillates from 4.2% of cases, to infected by the trematode species *Gorgodera varsoviensis* up to - 74.0% of cases to infected with the *Haplometra cylindracea* species. At the same time, the intensivity of invasion (I.I) with helminthes of the host is enough variable, and its values oscillate from - 1 ex. when the host is infected by the monogenean species *Polistoma integerrimum* up to 99 ex., when it is infected by the nematode of species *Tylodelphys excavata*.

The divergence of invasive values of the host species can be explained by the fact that the natural ecological factors are different during an annual cycle, but also its vital cycle. These factors as a whole favor the activity of the faunal diversity (intermediate, complementary, reservoir, definitive hosts), in a certain area and in a certain period of time participating in the development life cycle of specific helminthes species to *Rana dalmatina*.

The older the host, the more time it has had for the formation of biocenotic relationships in their specific ecosystems but and the possibility of infection with certain parasitic agents. Therefore, the extensivity and intensivity of the invasion of the agile frog, as well as the other species of amphibians investigated helminthologically on the territory of our country, changes so that the diversity of their helminthes species becomes richer, and the helminthological indices more amplified.

In addition, the changes of the helminthological indices and the structure of the helminth fauna related to age host are often proportional to their morpho-physiological changes, resulting in the behavior or the nutritional spectrum, which also contributes to a certain change in infection with various parasitic agents.

In order to appreciation the distribution of parasitic agents detected in amphibians, as well as to determine a possible infection with these species of helminthes of other groups of vertebrate animals, the structure of the helminthic fauna in four age categories of the population of *Rana dalmatina* egg - larvae - juveniles - adult forms were evaluated.

According to the results obtained, during the helminthological investigation of the *Rana dalmatina* eggs, species of helminthes they weren't established, while the presence of parasitic agents from 2 taxonomic classes (Secernentea, Trematoda) was established in its larvae and juveniles, but and in the adult forms of amphibians, the presence of four taxonomic classes (Secernentea, Trematoda, Palaeacanthocephala, Monogenea) was

established, with a different number of species (Figure 1).
Therefore, in the larvae of the *Rana dalmatina* species, four species of helminths was established, of which three species of trematodes (*Opisthioglyphe ranae, Haplometra cylindracea, Strigea sphaerula*) and one species of nematode (*Agamospirura* sp.), which constitutes 22.2% of the total species of detected helminthes.

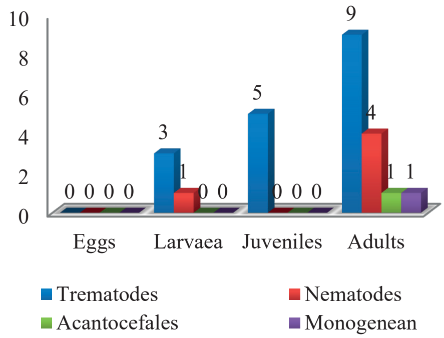

Figure 1. The structure of the helminthic fauna in agile frog species depending on its ontogenesis

In the juveniles, the presence of five helminth species was established, all of which are trematodes (*Opisthioglyphe ranae, Strigea sphaerula, Haplometra cylindracea, Telorchis stossichi*) which constitutes 27.7% of the total helminth species detected, and in the adult forms of amphibians the presence of nine trematode species (O. *ranae, H. variegatus, G. varsoviensis, G. vitelliloba, P. medians, D. subclavatus, S. sphaerula, H. cylindracea, T. stossichi*), four nematode species (*C. ornate, O. filiformis, O. duboisi, Rh. bufonis*), and a species of acanthocephalus (*A. ranae*) and a species of monogeneans (*P. integerrimum*) was established which constitutes 83.3% of the total species of helminthes detected in the host species.
According to the evaluation of the obtained data, it was determined that some detected helminth species are specific only for a certain ontogenetic period, such as the helminth species (*H. variegatus, G. varsoviensis, G. vitelliloba, P. claviger, P. medians, D. subclavatus, C. ornata, O. filiformis, O. duboisi, Rh. Bufonis, A, rane, P. integerrimum*) detected only in the adult forms of agile frog.

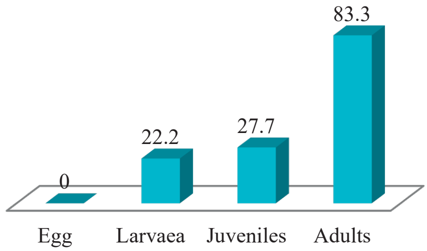

Figure 2. The structure of the helminthic fauna depending of the ontogeny of host

At the same time, the presence of helminth species that are specific to only one period of the host's ontogeny was determined, for example *T. stossichi* species in juveniles and *Agamospirura* sp. species in the stage of larval development. One and the same helminth species was established to be specific of the host in different ontogenetic periods of it. Thus 11.0% of the total helminth species detected are specific to both larval, juvenile as well as adult forms, 5.6 % of cases are specific to juveniles and adult forms of amphibians, larvae and juveniles, respectively juveniles, but the species specific only to adult forms of amphibians constitute 66.65% of the total species of helminthes detected in the host species (Figure 3).

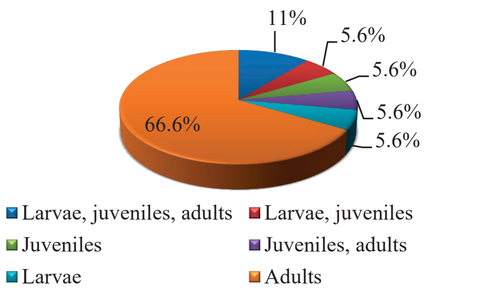

Figure 3. The degree of specificity of helminths depending on the ontogeny of host

To determine the diversity of the helminthic fauna in amphibians depending on their main phenological phases, these were investigated over the entire annual life cycle during the spring, summer, autumn seasonal successions.

As a result of the investigations, an evolution of the parasitic fauna of the *Rana dalmatina* species was recorded in its different phenological phases: the exit phase from the hibernation period, the movement towards the summer sites, the achievement of metamorphosis, the leaving of the summer sites and the initiation of their hibernation phase (Figure 4).

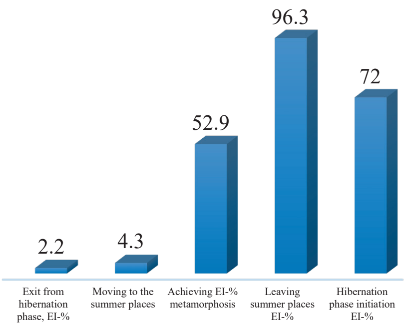

Figure 4. Degree of helminth infestation of *Rana dalmatina* species according to specific phenological phases

The recording of a poorer parasitic fauna in this amphibian species during the spring period (exit from the hibernation phase, orientation towards the breeding pools, breeding period) it is characterized by the absence of the obligatory intermediate hosts in the development cycle of helminth species specific for amphibian due to their unfavorable ecological optimum, as well as very low amount of food for the hosts.

In order to evaluate the degree of infestation with helminthes in the aspect of mono- and polyinvasions during an annual and vital cycle of amphibians, it was established that in the *Rana dalmatina* species the infestation in the aspect of polyinvasion are predominates (Figure 5).

In order to establish the role of the *Rana dalmatina* species in the formation and maintenance of foci of parasitic agents common to synanthropic, domestic, wild, companion animals and humans, complex research was carried out that allowed the determination of different stages of development (larval forms, adult forms) of all elements invasiveness detected in this species of ecaudate amphibians.

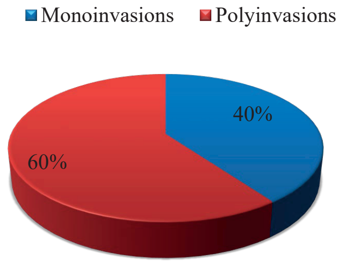

Figure 5. The structure of the helminthic fauna in *Rana dalmatina* species in terms of mono and polyinvasions

Thus, according to our helminthological investigations, as a result of which the presence of 18 species of helminthes was established, it was found that the predominant group is represented by trematodes (n = 11) which constitute 61.1% of cases, nematodes - 27.7% of cases (n = 5) and acanthocephals and monogeneans with 5.6% of cases (n = 1) each (Figure 6).

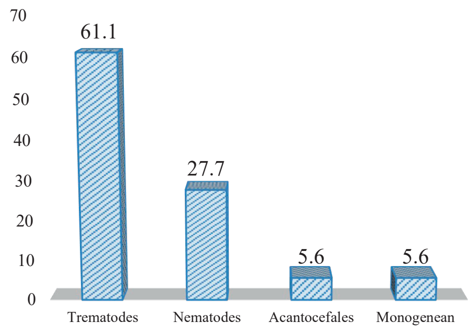

Figure 6. Indices of extensivity of taxonomic classes of helminths in amphibians

When evaluating the data and the invasive composition, it was found that the adult forms of helminthes are predominant for the larval forms, so that, when the host species is infested with trematodes 27.3% of cases are metacercariae and 72.7% of cases are the adult forms, when the host species is infested with nematodes 20.0% of cases are the larval stages, and 80.8% of cases are the adult forms, when infesting the host species with acanthocephales and monogeneans, it was established that the helminth species are an adult form, each constituting 5.6% of cases (Figure 7).

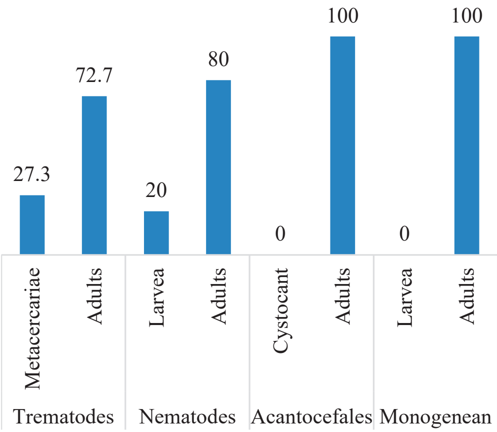

Figure 7. Helminthological indices of *Rana dalmatina* species depending on the ontogeny of the parasitic agents

Therefore, during the helminthological investigation of *Rana dalmatina* species, it was found that this host is predominantly infested with invasive elements of adult form.

According to specialized scientific works, the role of amphibians as vectors are mentioned (Gherasim, 2023a; Herczeg et al., 2021; Frolov et al., 2015; Buga et al., 2013). Analyzing our data referring to the diversity of helminth species in amphibians, determining the ontogenetic stages of the infected host, but also of the established parasitic agents, we can mention that the *Rana dalmatina*, host species, shows an increased degree of vectorization of her helminthic agents for wild, domestic, company animals and the human. This process is explained not only by the evolutionary trophic relations in the ecosystem (prey-predator), but also by the possibility of its simultaneous infestation with several species of parasitic agents.

Rana dalmatina species was characterized by an infestation with parasitic agents with up to 5 species of helminthes simultaneously. According to the assessments, it was determined that the specimens in 40.0% of cases were infested with a single species of helminthes, 10% of cases - with 2 species of helminthes, 20.0% of cases - with 3 species of helminthes, 20.0 % of cases - with 4 species of helminthes and 10.0% of cases - with 5 species of helminthes (Figure 8).

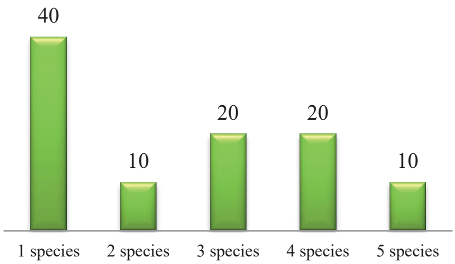

Figure 8. The degree of co-infection of the *Rana dalmatina* species

To evaluating the degree of vectorization of parasitic agents by the host species *Rana dalmatina* in the ecosystems of the Republic of Moldova, it was determined that of the total helminth species detected, 5.6% of the cases are specific to fish, 5.6% of the cases are specific to reptiles, 11.1 % of the cases are specific to birds and 77.7 are specific only to amphibians (Figure 9).

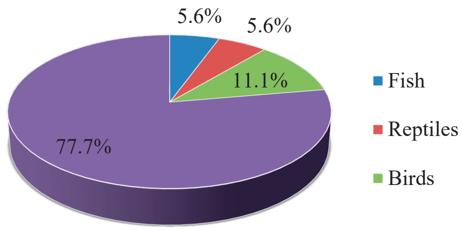

Figure 9. The degree of vectorization of parasitic agents by the *Rana dalmatina* species

So, the distribution and dynamics of the appearance of parasites in a special environment and time in the host species *Rana dalmatina*, as well as the factors that regulate the relationship between the host with parasite at the individual level, or at the population level, represent a complex study with a importance approach to various aspects biological, ecological and helminthological both of the host organism and of the parasite.

CONCLUSIONS

For the first time in the Republic of Moldova, helminthological investigations were carried out on the species of ecaudate amphibians *Rana dalmatina* Bonaparte, 1840.

It has been established the presence of 18 species of helminths, which from a taxonomic point of view fall into three phylums (Platyhelminthes, Nematoda, Acanthocephala), four classes (Trematoda, Monogenea, Secernentea, Palaeacanthocephala), 8 ordine (Plagiorchiida, Echinostomida, Diplostomida, Ascaridida, Strongylida, Rhabditida, Echinorhynchida, Polystomatida), fifteen families (Omphalometridae, Haematoloechidae, Gorgoderidae, Lecithodendriidae, Pleurogenidae, Diplodiscidae, Diplostomatidae, Strigeidae, Macroderoididae, Telorchiidae, Cosmocercidae, Molineidae, Rhabdiasidae, Echinorhynchidae, Polystomatidae) and seventeen genres (*Opisthioglyphe, Haematoloechus, Gorgodera, Gorgoderina, Pleurogenes, Pleurogenoides, Dipodiscus, Strigea, Haplometra, Tylodelphys, Telorchis, Cosmocerca, Oswalcocruzia, Agamospirura, Rhabdias, Acanthocephalus, Polystoma*)

The main helminthological indices were studied depending on the host age, but the obtained results allowed us to conclude that with the increase in the size and age of amphibians, the parasitic fauna increases as well as the degree of helminthes infestation. This appearance demonstrates the intensification of the feeding of adult amphibians and the accumulation of parasitic agents in their body from previous periods, as well as the increase in the size and diversity of food favoring the simultaneous breakthrough of a greater number of parasitic agents into the host's body, which leads to the increase in the diversity of the helminthic fauna.

The structure of the helminthic fauna was studied depending on the phenology of the host, which allowed the appreciation of the degree of knowledge of the period, stations and habitats where it is possible to vectorize of parasitic agents by the *Rana dalmatina* species to the definitive hosts of these helminthes, which is particularly important for the agile frog, which uses aquatic ecosystems only during the breeding season.

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SPATIAL - TEMPORAL DISTRIBUTION OF THE EURASIAN OTTER (*Lutra lutra*) POPULATION SOMOVA AQUATIC COMPLEX - PARCHEȘ, ROMANIA

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Abstract

The purpose of this study was to obtain information about otter population in terms of spatio-temporal distribution, abundance and/or density and other ecological characteristics of the otter population in the Somova - Parcheș aquatic complex. Because the otter is a solitary, nocturnal animal with a large distribution area, direct methods of assessing population numbers are difficult. That is why indirect methods were used in this study, such as: counting footprints, feces, latrines, territory marking places. The abundance of presence signs is considered a measure of the activity of the species. During the study period, a number of 256 signs of presence were inventoried (spraints, jellies, footprints) and 2 specimens of otter were identified (1 dead adult and a live chick both found in fishing nets). The greatest abundance of presence signs is found on the wooded inner hills of the lacustrine complex. Starting from the west to the east of the complex we can consider 4 areas with a high density of signs of presence. The most abundant signs of presence were found in the winter season (33.2%).

***Key words**: aquatic complex, eurasian otter, Somova - Parcheș, spraint.*

INTRODUCTION

The European otter is a top semi - aquatic predator, highly adaptable, which had a wide distribution across the Eurasian continent. Due to a complex of local and regional factors (such as habitat loss, pollution, and hunting) in the 1960s and '70s, populations of this species underwent a dramatic decline. Therefore, ongoing conservation efforts have been undertaken, either by restoring suitable habitats where populations have declined, or by reintroducing wild otters to areas from which they had disappeared.

Strict protection measures have led to some otter populations expanding and recovering naturally (Kruuk, 2006).

The Eurasian otter (*Lutra lutra*) is a territorial animal, marking and defending its territory against conspecific individuals. Their territories can vary depending on food availability and other vital resources. It is worth noting that otter territories can be affected by human activities, such as deforestation or water pollution, which can lead to conflicts between otters and humans or changes in their territorial behaviour. As semi-aquatic species, their distribution and density are influenced by all biotic and abiotic factors of both aquatic and terrestrial environments.

In Romania, the Eurasian otter is part of our country's wildlife. It is mainly found in mountainous and hilly areas, along rivers and streams with flowing water, where they find food and optimal habitats for shelter, reproduction, and raising their young.

It is recognized that the most important factor influencing the distribution and density of a wild population is food availability (Manly et al., 2002).

Legally, the European otter is a protected species in Romania, included in the annexes of the Bern Convention and the Habitats Directive. This entails conservation and habitat protection measures, as well as population monitoring to ensure their survival and well-being in the wild. Despite legal protection and conservation efforts, otters are still threatened by pollution, habitat degradation, territory fragmentation, and other disturbances caused by human activities. Thus, the continuation of conservation efforts and raising awareness of the importance of preserving natural habitats is crucial for the future of this species in Romania and across Europe. The aim of this study is to present aspects regarding the abundance and distribution of otter signs within the Somova -

Parcheș aquatic complex and to identify the main factors influencing this distribution.

This study used otter spraints densities to estimate the density of this population. Although this indirect method is less accurate and some authors consider it a poor method, it is still an effective method considering the elusive character of the otter.

The increasingly used environmental DNA (eDNA) appears effective to detect cryptic aquatic species (Yonezawa et al., 2020).

However, DNA degradation over time and the time lag between species presence and eDNA sampling can also lead to false negatives (Barnes et al., 2014).

Therefore, the search for presence signs remains a powerful method for detecting species presence (Lerone et al., 2015). Also, there are studies where it has been shown that there are high correlations between the number of genotyped individuals and the number of spraints ($r^2 = 72$, after Lanszki et al., 2008).

MATERIALS AND METHODS

Study area

The study was carried out between 2019 and 2022 and 252 transects were surveyed.

The study area is represented by the aquatic complex Somova - Parcheș, part of the Danube Delta included in the Danube Delta Biosphere Reserve. It is also part of the site of community importance ROSCI 0065 (Figure 1).

The Somova - Parcheș aquatic complex is an important ecosystem in Romania, located in Tulcea County, in the southeastern part of the country. This aquatic complex is part of the Danube Delta, one of the largest and best-preserved deltas in Europe.

Scientifically, the Somova - Parcheș aquatic complex is characterized by a variety of wetland habitats, including lakes, ponds, marshes, channels and streams, surrounded by reed and bulrush vegetation, as well as floodplain forests. These habitats provide optimal conditions for a wide diversity of plant and animal species adapted to aquatic environments.

The total area of the complex under free water circulation is S = 9170 ha.

The main types of habitats in the Somova - Parcheș aquatic complex are big lakes (19.3%), channels (3.8%), small lakes, shallow waters (~2.2%) (Török, 2006). The rest of the complex is covered predominantly by reeds (either in compact form or floating in association with trees and other vegetation) (~60%).

Also, 10.36% are forested areas, 2.72% meadows and 1.63% floating aquatic vegetation.

Data collection and analysis

For data collection, the linear transect method was employed to observe various otter signs along the banks of waterways (including both lakes and channels). The transects had a length of 1200 meters. Otter presence signs encountered (such as jellies, spraints, and footprints) along with coordinates were recorded on a field datasheet. Additionally, the freshness of the feces, habitat characteristics, distance of signs from the water's edge and the type of substrate on which the sign was identified (sand, rock, soil, tree trunk) were noted.

Figure 1. Map of the Somova - Parcheș aquatic complex and the spatial distribution of otter presence signs (processed in Google Earth Pro)

RESULTS AND DISCUSSIONS

A total of 256 presence signs of the studied specie were identified during the study period. The presence signs found included spraints, annal jellies, footprints and tracks in the substrate. Two specimens were also identified: a live cub caught in fishermen's nets (which was released) and a male specimen adult found dead and discarded on a brook (most likely found drowned in nets and then discarded by fishermen) (Figure 2).

Figure 2. Other signs of otter presence than spraints (original photos)

Spraints were the most abundant, found either individually or in latrines, representing 91.01% of the total signs. Footprints accounted for 4.69%, annal jellies for 2.34%, and tracks 1.17% (Figure 3).

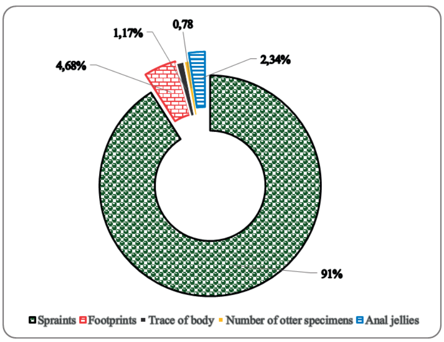
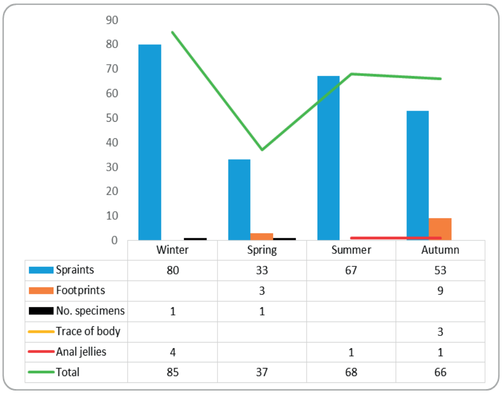


Figure 3. Abundance of presence signs

From the point of view of temporal distribution, the highest number of signs of presence was reported in winter and summer and the lowest number of signs in spring (Figure 4).



	Winter	Spring	Summer	Autumn
Spraints	80	33	67	53
Footprints		3		9
No. specimens	1	1		
Trace of body				3
Anal jellies	4		1	1
Total	85	37	68	66

Figure 4. Temporal distribution of presence signs

Somova - Parcheș is an aquatic complex with free water circulation.

The water coverage percentage of the complex depends on the levels of the Danube River.

The complex is flooded in the spring through the canals and backwaters that connect with the Danube and the main way of water circulation inside the complex is represented by the Somova backwater (an old branch of the Danube).

We consider the low abundance of otter presence signs in the spring was strongly influenced by this aspect because a large variation in the flow and water level leads to the washing of excrement (Charbonnel et al., 2015).

The two specimens were also found in the cold season (December, respectively the first days of March). The sub-adult specimen was found in the net by commercial fishermen (and released) and the adult specimen was found dead (by drowning) thrown on the water's edge (most likely it was also found in the fishing nets but was already dead).

The otter is an opportunistic species whose feeding behavior is determined by food abundance (Braña et al., 1993; Young et al. 2008) and according to optimal feeding theory it will hunt those prey that provide the most energy with the least search effort (Stephens & Krebs 1986). The opportunism of the species in terms of food procurement was highlighted by the fact that the identified specimens were found in fishermen's nets.

Based on the size of the otter tracks, which were measured and recorded, it was found that all the tracks belong to adult specimens (they were > 5 cm in size). The percentage of females was 58.33% and of males 41.67%. It is a high probability that it was included in the female population also some sub-adult males because at this age they have almost the same track size as the adult females (Bouroș, 2016).

44% of the total signs of presence were found on Lake Parcheș followed by Lake Câșla with 28.4%, Ivanova channel 17.69% and Lake Somova 9.87% (Figure 5).

Although, there are no significant differences in the abundance of signs of presence along the complex (from west to east) we can consider that we have three agglomerations of abundance: Lake Parcheș, Ivanova Channel, Câșla Lake (Figure 6).

Habitat features influence the abundance and availability of food, actually playing an important role in the ecology of this species, influencing: distribution, density, breeding period, reproduction success and mortality (Kruuk, 2006).

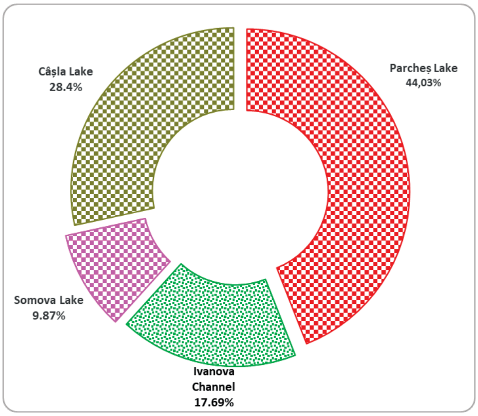


Figure 5. Abundance distribution of presence signs on the main lakes

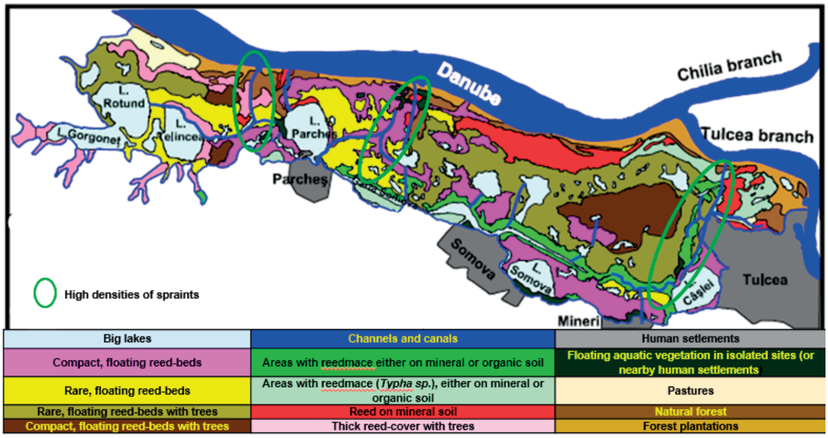


Figure 6. Spatial distribution of the main habitat types existing in the aquatic complex Somova - Parches (processed after Török, 2005)

By correlating the abundance of presence signs with the habitat types (Figure 6), we can see that they have the following characteristics in common:

- they are abundant on the brook and channels that maintain the connection between the aquatic complex Somova - Parches and the Danube River. This allows the otter to move easily between the lotic and lentic ecosystem depending on food availability. Remonti et al. (2008) found a positive correlation between fish consumption by otters, fish biomass and flow.
- the three aggregations are located in areas with a higher percentage of forest cover and woodland vegetation. Forests act as buffer zones, contributing to the improvement of physico - chemical water parameters (temperature, oxygen, pH). They also prevent the penetration of sunlight and precipitation, influencing the persistence of otter spraints (Biffi et al., 2019; Marcelli et al., 2012).
- all three zones are covered to a high percentage by dense floating reed beds, mixed with trees (or not), which, besides acting as

buffer and water purification zones, serve as feeding, resting, breeding, and nurturing shelters. Robitaille & Laurence (2002) demonstrated that areas covered with reeds have a strong positive influence on the densities of this species.

CONCLUSIONS

The Eurasian otter (*Lutra lutra*) is a top predator in aquatic ecosystems and plays a key role in its functioning. It is considered a bio-indicator species because its presence and abundance shows that it has something to eat and this means that that aquatic ecosystem has good water quality.

In the present study, the abundance of signs of presence demonstrates that the population of this species is well represented in the studied aquatic complex.

The aquatic complex Somova - Parcheș, through the multitude of habitats present, offers optimal conditions for the otter population both from the point of view of the surface of the complex but also of the vegetation and the abundance of food resources. The ichthyofauna of the complex is rich and diverse due to the permanent connection with the Danube River. Also, if the abundance of food resources and other conditions dictate it, the otter can easily migrate between the Somova - Parcheș aquatic complex and the Danube River.

In this complex he has no enemies except man. But human settlements are at a medium distance from its preferred habitats so this species can only come into conflict with commercial fishermen. In fact, the main anthropogenic impact on the population is only the interference between the activity of the fishermen and that of the otters (fishing gear placed on brooks and canals can lead to the catching and drowning of individuals).

Also, the high degree of coverage of the complex (with forest vegetation and reeds) positively influences the population of otters. Vegetated spaces are essential for otter populations that spend more than 50% of their time in woodland (Jefferies, 1986).

The Somova - Parcheș aquatic complex represents a vital aquatic environment for biodiversity conservation in the Danube Delta region and is essential for the survival and well-being of the otter population.

The protection and proper management of this ecosystem are particularly important for the conservation of nature and the maintenance of the ecological balance in the area.

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HELMINTH BIODIVERSITY AND HEAVY METAL CONTAMINATION OF *Perca fluviatilis* (Linnaeus, 1758) AND *Eustrongylides excisus* (Jägerskiöld, 1909) LARVAE FROM THE WETLAND MANDRA-PODA

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Abstract

The study presents the first data on the biodiversity of helminths and helminth communities of European perch (Perca fluviatilis Linnaeus, 1758) from the freshwater ecosystem of the anthropogenically affected protected area Mandra-Poda. Thirty specimens of perch were examined by the method of complete helminthological study. The infection characteristics and the dominant structure of the helminth communities were determined. Helminth communities were analyzed at two levels: infracommunities and component communities. Basic biotic indices are presented. The core species in the helminth communities of perch is Eustrongylides excises Jägerskiöld, 1909 larvae (Nematoda; Dioctophymatidae) (P% = 60; MI = 7.12; 1-51 specimens). New data on the content of heavy metals and their circulation in the system water-sediment-perch and its helminth Eustrongylides excisus have been established. New data on the bioindicator significance of helminths and helminth communities of European perch are discussed.

Key words: *bioindication, Black Sea Water Basin, european perch, ecological indices, Mandra-Poda.*

INTRODUCTION

Mandra-Poda complex is a part of the Burgas Wetlands, Black Sea Basin Region, Ecoregion 12: Pontic Province, Sub-Ecoregion 12-2: Black Sea, Southeast Bulgaria. It is located south of the industrial zone of the city of Burgas. According to the Ramsar Convention, the Mandra-Poda complex has been declared a wetland of international importance. The Mandra-Poda complex is a protected area under the Habitats Directive (Directive 92/43/EEC) and the Birds Directive (79/409/EEC), as well as the Corine Biotope, an Ornithologically Important Site. Izvorska, Fakiyska, Sredetska, and Rusokastrenska rivers flow into the Mandra Lake. The Lake is located in a river valley, across the sea coast. The main economic activities in the Lake are related to the use of water by Lukoil Neftohim for industrial needs. The main threats are related to significant anthropogenic pressure from the construction of areas along the coast of the wetland and the destruction of the natural habitats located there (especially wetland meadows), eutrophication of the wetland, excessive water use, development of the surrounding infrastructure, pollution with chemicals and solid municipal waste from the watershed, overfishing, poaching, significant disturbance, others. On the territory of the complex, three protected areas have been declared, according to the Law for the Protected Areas (‘Poda’; ‘Ustie na reka Izvorska’; ‘Uzungeren’), for conservation of the habitats of endangered and rare bird species. The complex is part of the migration flyway "Via Pontica". Although the freshwater ecosystem is under serious anthropogenic interference, the Mandra-Poda complex is important for biodiversity conservation. Research on parasites and parasite communities of freshwater fish from the Mandra-Poda area was carried out only by Margaritov (1959). There are single scientific studies on chemical indicators of water pollution and the content of pollutants in the muscles of freshwater fish species (Georgieva et al., 2015; Peycheva et al., 2022). The study aims to present research results on helminths and helminth communities of *P. percae* from the Mandra-Poda complex and monitor Se concentrations in waters, sediments, tissues, and organs of European perch and its parasite *E. excisus*.

MATERIALS AND METHODS

In 2023, thirty specimens of *Perca fluviatilis* from the Mandra-Poda Complex were examined for helminths and Selenium (Se) content. The examined fish were caught by gill nets (BSS EN 14757:2015 Water quality. Sampling of fish with multi-mesh gillnets), according to permission from the Ministry of Agriculture of the Republic of Bulgaria. The scientific name is represented according to the FishBase database (Froese and Pauly, 2020). The fish were collected in the Northeastern section of the Complex (42°24'12.31″N, and 27°19'18.05"E; 309 m; Figure 1).

Figure 1. Studied biotopes from the Complex Mandra-Poda

The helminthological study of *P. Fluviatilis* was carried out according to Petrochenko (1956); Zashev & Margaritov (1966); Bauer (1987); Moravec (2013). Collected helminth specimens were fixed in 70% of ethyl alcohol. Species diversity was determined on permanent slides according to the method of staining with iron acetocarmine (Georgiev et al., 1986; Sholz and Hanzelova, 1998) and on temporary slides carried out by the methods of Moravec (2013) and Petrochenko (1956). Helminth community structure was analyzed on two levels: on the level of intracommunity (total number of fish species; total and mean number of fish specimens; Brillouin's diversity index (HB) and on the level of component community (prevalence (P%); mean intensity (MI)). Helminth species in the component community were divided into three groups: core species (P%>20), component species (P%>10), and accidental species (P%<10), according to the criteria of Magurran (1988); Bush et al. (1997), and Kennedy (1997).

Tissue and organ samples of *P. fluviatilis* and *E. excisus* were prepared for determination of Se content according to Nachev et al. (2013). The pre-weighed and thawed samples of liver, muscle and skin of *P. fluviatilis* (to 300 mg wet weight) as well as *E. excisus*, extracted from the cysts (to 100 mg wet weight) were subjected to acid digestion with aqua regia and microwave heating - Method B: "Microwave heating with temperature control at 175±5°C". Samples of 2 g have been used in closed vessels under pressure with 6.0 ml HCl and 2.0 ml HNO_3 and determination of the element using ICP-OES, according to EN ISO 16170:2016. In surface water and sediment samples, selenium concentrations were determined according to EN ISO 11885:2007

Water quality - Determination of selected elements by inductively coupled plasma optical emission spectrometry (ICP-OES) and EN 16170:2016 Sludge, treated biowaste and soil - Determination of elements using ICP-OES.

Bioconcretacion factors were calculated to determine the accumulation capacity of muscle, skin, and liver of European perch and E. excisus from the freshwater environment (Bioconcentration factor, $BCF=C_{SeSkin/SeLiver/SeMuscle/Se\mathit{E.excisus}}/C_{SeWater/SeSediments}$) (Sures et al., 1999), and accumulation capacity of *E. excisus* from organs and tissues of the European perch (Bioaccumulation factor, $BAF=C_{Se\_E.excisus}/C_{Se\_fish\_tissues\_organs}$) (Zaharieva, 2022a). Spearman rank correlation (r_s) was calculated to check the relationship between Se concentrations in samples of environment (water, sediments), in the samples of liver, muscles, and skin from fish hosts, and in samples of its nematode species *E. excisus* (Sokal and Rohlf, 1981). The coefficient of determination (r_s^2) is also presented, which reflects what percentage of the factor variable will cause changes in the outcome variable. A Friedman test ANOVA was applied to determine the significance of differences between the selenium content in the studied fish tissues and organs and *E. excisus* (Sokal & Rohlf, 1981). The results were statistically processed using Statistica 10 (StatSoft Inc., 2011) and MS Exel (Microsoft 2010).

RESULTS AND DISCUSSIONS

Characteristics of the studied fish species

Perca fluviatilis Linnaeus, 1758 (Percidae) is a freshwater, demersal, and brackish fish species. The European perch is a predatory species. It feeds mainly on zooplankton, zoobenthos, and small fish. The species is defined as not endangered in the International Red Book (LC; IUCN) (Kottelat & Freyhof, 2007; Froese & Pauly, 2020; Karapetkova & Zhivkov, 2006). The European perch is not a protected species on the territory of Bulgaria. The species is widespread in different types of water bodies in the country. *P. fluviatilis* could be of more economic importance but is mainly subject to recreational fishing. Maximum length (cm) and weight were determined for each examined specimen (g). The maximal length of studied specimens varies from 15-21 cm (17.78±1.28), and the weight from42-72 g (56.1±8.55).

Helminths and Helminth community structure

In 2023, as a result of the ecologoparasitological examinations of 30 specimens of European perch, *Perca fluviatilis* Linnaeus, 1758 from the Mandra-Poda Complex, three taxa of endohelminths was established: *Proteocephalus percae* (Gmelin, 1790) La Rue, 1911; *Acanthocephalus lucii* (Müller, 1776) *Eustrongylus excisus* (Jägerskiöld, 1909), larvae, belonging to three classes, three orders, three families and three genera (Table 1).

Table 1. Biodiversity and ecological indices of helminths and helminth communities of *Perca fluviatilis* from the Mandra-Poda Complex

Perca fluviatilis ($N^1 = 30$) Helminth species	n^2	p^3	P%[4]	MI[5] (min.-max.)
Class CestodaRudolphi, 1808 **Order** Tetraphyllidea (Beneden, 1849) Carus, 1863 **Family** Proteocephalidae La Rue, 1911 **Genus** *Proteocephalus* Weinland, 1858				
Proteocephalus percae juv. (Gmelin, 1790) La Rue, 1911	2	2	6.67	1 (1)
Class AcanthocephalaRudolphi, 1808 **Order** Echinorhynchida Southwell et Macfie, 1925 **Family** Echinorhynchidae (Cobbold, 1879) Hamann, 1892 **Genus** *Acanthocephalus* Koelreuther, 1711				
Acanthocephalus lucii (Müller, 1776)	2	2	6,67	1 (1)
Class Nematoda Rudolphi, 1808 **Order** Dioctophymida (Skrjabin, 1927) Schulz et Gvosdev, 1970 **Family** Dioctophymidae Railliet, 1915 **Genus** *Eustrongylides* Jägerskiöld, 1909				
*Eustrongylus*excisus (*Jägerskiöld, 1909*), larvae	18	128	60	7.12 (1-51)

Legend: [1]N = total number of examined fish specimens.
[2]n = total number of infected fish specimens.
[3]p = total number of helminth specimens.
[4]P% = prevalence.
[5]MI = mean intensity.

***Proteocephalus percae* (Gmelin, 1790) La Rue, 1911** is an intestinal parasite of *Gymnocephalus cernua* (Linnaeus, 1758); *P. fluviatilis; Esox lucius* Linnaeus, 1758, etc. The developmental cycle is not well understood, but plerocercoids have been reported to develop in representatives of the genus *Cyclops* Müller, 1785 (Bauer, 1987; Kakacheva-Avramova, 1983). *Pr. percae* was reported as parasite species of *P. fluviatilis* from Lake Srebarna (Shukerova, 2010; Shukerova et al., 2010); from the Maritsa River (Kuzmanova et al., 2019; Kuzmanova et al., 2023) and the Danube River, near village of Kudelin (Zaharieva, 2022).

***Acanthocephalus lucii* (Müller, 1776)** develops as an adult in many freshwater fish species from Cyprinidae, Salmonidae, Percidae, Siluridae, etc. The intermediate host is *Asellus aquaticus* (Linnaeus, 1758) (Bauer, 1987; Kakacheva–Avramova, 1983). *A. aquaticus* is a bioindicator for α-mesosaprobity and is in Group D of tolerant forms regarding environmental conditions in habitats (Belkinova et al., 2013). *A. lucii* was reported as parasite species of *P. fluviatilis* from Lake Srebarna (Shukerova, 2010; Shukerova et al., 2010); from the Danube River (Matgaritov, 1966; Kakacheva et al., 1978), and the Maritsa River (Kuzmanova et al., 2019; Kuzmanova et al., 2023).

***Eustrongylus excisus* (Jägerskiöld, 1909)** in the adult state parasitizes the glandular stomach of cormorants [*Phalacrocorax carbo* (Linnaeus, 1758), *Ph. pygmaeus* (Pallas, 1773)].It develops with the participation of two intermediate hosts.The first intermediate host is aquatic oligochaetes [*Lumbricus variegatus* (Müller, 1774), *Tubifex tubifex* (Müller, 1774), *Limnodrilus* sp.]. The localization of the larvae is initially in the intestine and then in the body cavity (larvae I stage), after which the larva of the second stage passes into the abdominal blood vessel, and the larva of the third stage with the bloodstream passes to the head and tail end of the host. A second intermediate host is various species of benthic fish [*Ponticola kessleri* (Günther, 1861), *Neogobius melanostomus* (Pallas, 1814), *Rutilus rutilus* (Linnaeus, 1758)]. Invasion occurs through food.In the organism of the host fish, the larvae of the III stage are localized in the body cavity or curled up in a circle under the serous coating on the surface of the internal organs. After another molt, they migrate into the musculature of the fish (most often the stomach and less frequently the dorsal). The IV-stage larva is coiled, encapsulated, and invasive. Reservoir hosts of *E. excisus*are: *P. fluviatilis*, *Leuciscus aspius* (Linnaeus, 1758), Silurus glanis Linnaeus, 1758, *E. lucius*, *Sander lucioperca* (Linnaeus, 1758), *Sander volgensis* (Gmelin, 1789), *G. cernua*, *Chalcalburnus chalcoides* (Güldenstädt, 1772), *Bentophilus macrocephalus* (Pallas, 1787), *Huso huso* (Linnaeus, 1758), *Acipenser ruthenus* Linnaeus, 1758, *A. gueldenstaedtii* Brandt & Ratzeburg, 1833, *Leuciscus idus* (Linnaeus, 1758), *Luciobarbus brachycephalus* (Kessler, 1872), *Pelophylax ridibundus* (Pallas, 1771), *Natrix tessellata* (Laurenti, 1768) (Bauer, 1987; Kakacheva-Avramova, 1983). *E. excisus* was reported as parasite species of *P. fluviatilis* from the Lake Srebarna (Shukerova, 2010; Shukerova et al., 2010; Hristov, 2013; Kirin et al., 2013a); from the Arda River (Kirin et al., 2013b); from the Danube River (Atanasov, 2012; Kirin et al., 2013a). *E. lucius* has been reported for the Mandra-Poda Complex from *S. lucioperca* as a reservoir host and from *Gobius* sp. as an intermediate host (Margaritov, 1959).

Component community

The presented helminth taxa were found in 18 of the studied 30 specimens of European perch (60%). Prevalence (P %), mean intensity (MI), and rank were determined for each taxon. *E. excisus* (P% = 60) is a core species of the endohelminth communities of *P. fluviatilis* from the Mandra-Poda Complex. The other two species are accidental ($P\%_{Pr.percae}$ = 6.67; $P\%_{Ac.lucii}$ = 6.67). *E. excisus* is was distinguished with the highest mean intensity (MI = 7.12). The mean intensity for the other two species is low ($MI_{Pr.percae} = MI_{Ac.lucii}$ = 1.0). Only two *Pr. percae* and *Ac. lucii specimens* are fixed in the infected specimens of *P. fluviatilis*. *E. excisus*is an allogenic species. *Pr. percae* and *Ac. lucii* are autogenic species. The found taxa are generalists for the helminth communities of *P. fluviatilis* from the Mandra-Poda Complex, Bulgaria (Table 1).

Infracommunity

The twelve examined specimens of *P. fluviatilis* are free of helminths (40%). A mixed invasion of *E. excisus* and *Ac. lucii* was found in two host specimens (6.67%) and in one of the studied specimens of European perch, a mixed invasion of *E. excisus* and *Pr. Percae* (3.34%). The maximum number of helminths found in a single specimen by the host is 51 (*E. excisus*). The average number of all endohelminth specimens is 7.34. The value of Brillouin's diversity index (HB) is low (HB = 0.121) (Table 2).

Table 2. Infracommunity data

Number of helminth species			
Number of infected fish	12	15	3
Number of helminth species	0	1	2
Number of helminth specimens			
Total number	132		
Mean±SD	7.34±14.25		
Range	1-51		
Mean HB±SD	0.121±0.03		

Concentration of Selenium (Se) in the system Water – Sediments - *P. fluviatilis* - *E. excisus*

The content of Se was determined in samples of skin, muscles, and liver of *P. fluviatilis* from the Mandra-Poda Complex, as well as in samples of *E. excisus* as a core species in the helminth communities of the European perch (wet weight and dry weight, mg.kg^{-1}). The highest content of Se was found in *E. excisus*, followed by that in liver samples of *P. fluviatilis* as wet weight. The content in water samples is the lowest, followed by that in muscle and skin as wet weight. The Se content of *E. excisus* was 1.46 times that of the perch liver, 4.91 times higher than that in the skin, 7.79 times more than in the perch muscles, and 753.34 times more than in the water samples (Table 3).

When comparing the results in dry weight, the highest content was reported in sediments, followed by that in *E. excisus* and the liver of European perch.

The lowest Se content was again reported in the muscle samples as dry weight. In these cases, the selenium content is the highest in the sediments.The Se content of the parasite was 3.92 times lower than that in the sediments and 1.42 times higher than that in the liver, 9.46 times higher than that in the skin, and 15.28 times higher than that in perch muscle as dry weight (Table 3).

Significant differences were found between Se concentrations in E. excisus and those in the skin, muscle, and liver of P. fluviatilis, waters, and sediments (Friedman's ANOVA, $p < 0.05$).

Table 3. Concentration of Selenium in tissues and organs of fish and its nematode species *E. excisus*

Samples	Se min.-max. mg.kg^{-1}	SeMean±SD
Skin (wet weight) *P.fluviatilis*	0.39-0.56	0.46±0.11
Muscle (wet weight) *P. fluviatilis*	0.15-0.44	0.29±0.20
Liver (wet weight) *P. fluviatilis*	1.28-1.79	1.54±0.36
E. excisus(wet weight) *P. fluviatilis*	2.16-2.36	2.26±0.14
Water	0.003-0.003	0.003
Skin (dry weight) *P. fluviatilis*	0.77-1.07	0.92±0.21
Muscle (dry weight) *P. fluviatilis*	0.29-0.84	0.57±0.39
Liver (dry weight) *P. fluviatilis*	5.11-7.15	6.13±1.45
E. excisus (dry weight)	6.36-11.05	8.71±3.32
Sediments	35.4-40.2	34.1±6.84

BCF comparing samples as wet weight for Se content was highest for the ratio of Se concentrations in *E. excises* to those in water samples, followed by that obtained between Se concentrations in liver and water samples, ofskin and water samples, and lastly BCF, obtained as a ratio between Se concentrations in muscle and water samples (Table 4).

Table 4. Bioconcentration factor (BCF), Bioaccumulation factor (BAF), and Spearman correlation coefficient (r_s) between the content of Se in water, sediments, *P. fluviatilis* and *E. excisus*

Fish – Parasite - Water	**BCF**	**r_s**	**p**
Skin (wet weight) - Water	154.34	-1****	<0.001
Muscle (wet weight) - Water	97.67	-1****	<0.001
Liver (wet weight) - Water	514.67	-1****	<0.001
E. excisus(wet weight) - Water	753.34	-1****	<0.001
Fish – Parasite - Sediments	**BCF**	**r_s**	**P**
Skin (dry weight) - Sediments	0.03	1****	<0.001
Muscle (dry weight) - Sediments	0.02	1****	<0.001
Liver (dry weight) - Sediments	0.18	1****	<0.001
E. excisus (dry weight) - Sediments	0.26	1****	<0.001
***E. excisus* - Fish**	**BAF**	**r_s**	**P**
E. excisus (wet weight) - Skin (wet weight)	4.88	1****	<0.001
E. excisus (wet weight) - Muscle (wet weight)	7.71	1****	<0.001
E. excisus (wet weight) - Liver (wet weight)	1.46	1****	<0.001
E. excisus (dry weight) - Skin (dry weight)	9.43	1****	<0.001
E. excisus (dry weight) - Muscle (dry weight)	15.38	1****	<0.001
E. excisus (dry weight) - Liver (dry weight)	1.42	1****	<0.001

Legend: **** - very significant correlation

BCF comparing samples as a dry weight for Se content was highest for the ratio of Se concentrations in *E. excises* to those in sediment samples, followed by that between samples of liver and sediments, skin and sediments, and in last place, between the concentration of Se in samples of muscle and sediments. Therefore, the wet-weight samples maintain the trend (Table 4).

BAF for tracking the accumulation capacity of *E. excisus* is also presented.BAF was highest for the ratio of Se content in the parasite to that in the muscle samples and lowest for the ratio of Se content in the parasite to that in the liver (Table 4).

Spearman rank correlation coefficient was determined to assess the possible relationships between concentrations of Se in water - sediments - fish tissues and organs - *E. excisus*. Very significant negative correlations were found between the Se content of the water samples and the corresponding samples of the parasite, liver, skin, and muscles.Therefore, as the concentrations of Se in the samples from the biological sites increase, the concentrations of Se in the water samples from the Mandra-Poda Complex decrease.All other relationships are positive, also with very significant correlations. Therefore, with increasing concentrations of Se in sediments, its concentrations in biological samples also increase, and with its increase in the organs and tissues of perch, their increase in *E. excisus* samples, respectively (Table 4).

The coefficient of determination (r_s^2) for all variants is 100%. Therefore, any changes in the content of selenium in the habitat of the perch (water, sediments) will lead to changes in the content of selenium in the tissues and organs of both the European perch and its parasite *E. excisus*.

DISCUSSION

A total of 30 specimens of European perch were examined, and three species of helminths were reported.*E. excisus* is a core species for the helminth communities of *P. fluviatilis*.*The present study used P. fluviatilis* and its parasite *E. excisus* as models to trace Se content and bioaccumulation potential.Selenium is a non-metallic element.It is an essential trace element necessary for the mineral nutrition of animals.Regardless, however, when slightly exceeding the required concentrations, it can cause toxic effects.Sources of water pollution with selenium are industry and agriculture due to the direct or indirect discharge of selenium-laden wastewater and soils with high selenium content through irrigation water or drainage water passing through coal ash repositories.Organisms readily absorb Se, so it can quickly reach toxic concentrations dangerous to fish and wildlife.Increasing Se concentrations lead to young and adult organisms' death or reduced reproduction.Se concentrations of 0.002-0.005 mg.l^{-1} water have been found to cause toxic and reproductive problems in fish.Sediments are temporary reservoirs of Se (Lemly, 1987).In the body of plants and animals, Se is mainly bound to proteins. Therefore, foods with a high protein content also contain high concentrations of Se (e.g., meat, seafood). The recommended daily intake of Se for humans is 0.026-0.035 mg/day (FAO/WHO, 1998); from 2000 years, it has been 0.055 mg/day (NAS, 2000).The upper limit for Se is 0.4 mg/day (FAO/WHO, 1998; NAS, 2000; UK EGVM, 2002).The lowest adverse effect level was 0.003 mg/day.Alkaline, poorly aerated environments favor the formation of selenites and selenates, which are water-soluble and easily absorbed by organisms (WHO, 2011).

According to Compilation of Legal Limits for Hazardous Substances in Fish and Fishery Products (FAO Fish.Circ.,(764)), permissible value for Se in foodstuff is 0.3 mg.kg^{-1}. According to Regulation No.18 of 27.05.2009 on the quality of water for irrigation of agricultural crops, in Bulgaria, the maximum permissible value for Se in water used to irrigate agricultural crops is 0.01 mg.l^{-1}.

Scientific research on Se content in the system Waters - Sediments - Freshwater fishes and their parasites is extremely limited. Even fewer are these for *P. fluviatilis* and *E. excisus*.Nachev et al.(2013) compared the capacity between *Pomphorhynchus laevis* (Müller, 1776) Porta (1908) and *Eustrongylides* sp., helminths of *Babus barbus* (Linnaeus, 1758) from the Danube River.As in the present study, they found much higher levels of Se in *E. excisus* than in the tissues and organs of the fish (*B. barbus*).According to the authors, the degree of accumulation is closely related to the parasites' taxonomic affiliation and stage of development.According to this study, an important factor is also the species of host and his lifestyle, i.e., in the tissues and gills of the European perch studied by us, Se concentrations are much higher than those in *B. barbus* from the Danube River, as well as in *E. excisus* with host *P. fluviatilis* from the Mandra-Poda Complex. Nachev et al. (2013) also indicate intestinal acanthocephalans mainly accumulate toxic elements, while *E. excisus* mainly accumulates essential elements, including Se. They state that *P. laevis* and *Eustrongylides* sp. can be used as bioindicators of metal pollution. Hursky and Pietrock (2015) investigated the effects of parasitism on Se bioaccumulation in juvenile *Oncorhynchus mykiss* (Walbaum, 1792). They found low Se bioaccumulation in *O. mykiss* infected with the nematode *Raphidascaris acus* (Boch, 1779). Nuutinen & Kukkonen (1998) found that with increasing Se concentrations in lake sediments, the accumulation of methylmercury (MeHg) in the body of oligochaetes (*Lumbriculus variegatus* Müller, 1774) decreased. The authors found that Se concentrations of 15 and 50 $mg.l^{-1}$ in sediments reduced Se accumulation in *L. variegatus* by 75, and 86%, respectively.

CONCLUSIONS

The three species of helminths (*P. percae*, *A. lucii*, and *E. excisus*) are reported for the first time for the helminth fauna of European perch from the Mandra-Poda Complex. *P. percae*, *A. lucii* are reported for the first time for the helminthfauna of the freshwater fishes from the complex. The Mandra-Poda Complex is a new locality for them. The obtained high concentrations of Se and the very significant correlation dependences give grounds for *E. excisus* to be used as a bioindicator of Se pollutionin the biomonitoring systems.

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PHYSICOCHEMICAL WATER PARAMETERS - LIMITING FACTORS ON THE RAINBOW TROUT GROWTH IN RECIRCULATING AQUACULTURE SYSTEMS

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Abstract

The rainbow trout raised in the recirculating system is dependent on the water quality, which must have certain specific parameters. Using specialized literature, the current research aimed at improving the rainbow trout growth conditions in different situations was approached and analyzed. The water parameters differ depending on the season, feed, photoperiod, filter system. The obtained results showed that the most important limiting factors of the physicochemical parameters of water are: temperature, dissolved oxygen, ammonia, nitrites, pH and turbidity. Trout farming in recirculating systems involves high energy consumption and high equipment cost. By optimizing, monitoring and strictly complying with the water physicochemical parameters in RAS, it is possible to improve water quality and obtain large productions of fish, which can quickly amortize the initial investments and profit gain.

Key words: *aquaculture, limits, management, RAS, water chemistry.*

INTRODUCTION

In recirculating aquaculture systems, the growth rate of trout is a complicated process that depends on external and internal factors affecting the trout. External environmental conditions such as feed quantity and quality, water physicochemical parameters and the way the system is designed affect the growth rate and welfare of the fish. The quality of fish production in aquaculture is closely linked to the well-being of fish (Broom, 1998; Southgate & Wall, 2001). Growth rate and well-being is also closely related to the internal physiological state of the trout, such as health, stress and reproductive system. In order to avoid stress and increase the well-being of the fish, care must be taken that the physicochemical parameters are interconnected according to the physiological needs of the trout.

All these processes of physicochemical parameters interconnection take place in the water. As a result, water is one of the limiting factors of crucial importance, and the physicochemical processes occurring within the water tip the balance between failure and success in a recirculating rainbow trout rearing system.

In recirculating systems, the need for make-up water is approximately 1000 L kg^{-1} forage that is 10^2 times less than the classic system (Blancheton et al., 2007).

The growth, development and reproduction of salmonids involves knowing the minimum and maximum values of the parameters, specific to the species of interest, as well as the application of effective management regarding production (Cocan et al., 2018).

In order to maintain the quality of the water, the quality parameters must be permanently checked. In general, water samples are collected manually, to be sent to the laboratory where they are analyzed, but the lost time does not allow rapid intervention on fish (Pasika & Gandla, 2020). The researchers differ on the frequency of parameter control. Therefore, some researchers think that the turbidity and the pH can be measured weekly, while temperature and oxygen is good to be measured every day (Ferreira et al., 2011).

Wi-fi equipment and sensors come to the aid of aquaculture so the parameters of the pools can be tracked even if you are not present near the pools (Jamroen et al., 2023; Lindholm-Lehto, 2023). It is very important to follow the parameters of the water qualitatively for trout and to intervene in time when the water degrades and endangers the life of fish.

MATERIALS AND METHODS

In order to write this article, papers were studied in which various physicochemical parameters of water were monitored. The study tried to discover and synthesize some of the most recent research on the physicochemical parameters of water from the specialized bibliography.
This study can form a basis for future research aimed at growing rainbow trout in recirculating systems.

RESULTS AND DISCUSSIONS

Temperature, Dissolved Oxygen and Ozone

Water temperature and dissolved oxygen content are closely related and can have a negative influence on fish growth (Matthews & Berg, 1997; Uiuiu et al., 2020). When temperature approaches the limit value, the oxygen absorption capacity in the trout's body decreases (Pörtner & Giomi, 2013).
Jiang et al. (2021), investigated the increase in antioxidant capacity to stress, how amylase, prosthesis and digestive lipase act, physiologic growth parameters, but also the growth factor similar to insulin in the liver, in conditions of temperature changes and dissolved oxygen. In this study trout were bred with 4.2 mg kg^{-1} and 9.6 mg kg^{-1} dissolved oxygen, at 21°C, 17°C, 13°C. The results showed that the increase in relation to the initial weight and use of the feed, decreased to 21°C and increased to 17°C.
The problems with rising temperatures were moderate in the presence of increased dissolved oxygen content. When the dose of dissolved oxygen was enlarged, survival increased, but 4 mg kg-1 of dissolved oxygen is the lowest survival level for trout. Regarding trout growth indicators, were decreased to 21°C and raised to 17°C.
According to specialized literature, trout feel best at 16-17°C, but Cocan (2008), in his research, concluded that the comfort temperature for trout is 12-14°C. Also, according to Mishra et al. (2020; 2021), trout grown at 14-16°C showed the best specific growth rate value.
Dissolved oxygen plays a key role in recirculating systems. (Ebeling & Timmons, 2012). Low oxygen reduces weight gain and decreases feed conversion (Chabot & Dutil, 2005; Tran-Duy et al., 2005). The lack of dissolved oxygen increases ammonia toxicity in rainbow trout (Thurston et al., 1981).
Water turbidity, NH_2, lack of oxygen, water color and dissolved or total organic carbon can be improved with ozone (Goel et al., 1995; Tango & Gagnon, 2003; Summerfelt, 2003). Other research has shown that using ozone can also be effective in fighting algae and some heavy metals (Langlais et al., 1991; Plummer & Edzwald, 2002). The reintroduction of ozone promotes the formation of dissolved oxygen (Summerfelt, 2003).
When ozone is used longer than necessary, there is a risk of increased mortality (Summerfelt et al., 2004).
The application of ozone in RAS could solve some problems regarding optimization of press parameters and fish growth in recirculating systems (Davidson et al., 2011). Ozone reduces total suspended solids, therefore increasing water transparency solar penetration is favored.
Davidson et al. (2011), propose the use of ozone in recirculating systems due to the increase of water quality.
Recirculating systems are subject to an increased risk of disease and parasite infestation. The most common method of disease control consists in the use of ultraviolet rays and ozone (Cocan, 2008). In general, ozone positively influences fish survival.

Heavy Metals

Heavy metals such as aluminum become increasingly bioavailable as pH decreases (<6.0) and the increase of free ionic calcium. Therefore, toxicity occurs at a low pH than at neutral or high pH (Playle & Wood, 1989). Therefore, fish diseases occur which can be attributed both to the decrease in pH and to the increase in the level of heavy metals which can cause the appearance of white spots on the mouth, gills and tegument.

Hunn & Schnick (1990) observed that a decrease in pH combined with heavy metals increases hyperexcitability and attempts by fish to get out of the water (swim to shore).

Majlesi et al. (2019), did research tracking mercury, cadmium and lead levels in muscle tissue in rainbow trout. The determinations showed concentrations of: mercury 0.22 mg/kg, cadmium 0.105 mg/kg and lead 1.070 mg/kg. The level of mercury and cadmium fell within the maximum levels allowed by the WHO (World Health Organization), but lead did not. Consumption of fish in the study area had no negative effects on human health. The release of wastewater, pollutants from human activities and certain feeds increases the level of heavy metals in trout (Majlesi et al., 2019).

Water pH and Nitrogen-based Compounds

The Directive of the Council of Europe of 06.08.2006 regarding the quality of fresh waters, which must be protected or improved in order to maintain fish life, stipulates that the pH should fall between 6 and 9, non-ionized ammonia in salmonid and cyprinid waters can have in concentrations of 0.005 mg/L (indicative) and 0.025 mg/L (mandatory), total ammonium (NH_3+NH_4) $\leq$ 0.04 mg L^{-1} and mandatorily $\leq$=1 mg L^{-1} and $NO_2 \leq 0.01$ mg L^{-1} (https://eur-lex.europa.eu).

The factor that determines the ratio of ammonia to ammonium in water is the pH. The activity of ammonia is influenced by the ionic strength and the temperature of the solution (https://ro.hach.com/parameters/ammonia).

Ammonia and ammonium (NH_3 and NH_4) concentrations are expressed in mg/L or PPM of N. From a chemical point of view, the relationship between ammonia and ammonium is: $NH_3 + H_2O \leftrightarrow NH_4^+ + OH^-$.

When pH decreases ammonium increases and ammonia decreases, and when pH increases ammonium decreases and ammonia increases. The activity of ammonia in water is much lower at low temperatures. As pH and temperature increase, so does ammonia. Knowing the temperature and pH, the percentage of ammonia (NH_3) in the total ammonia can be determined according to Table 1 (Emerson et al., 1975).

Table 1. The percentage of non-ionized ammonia according to pH and temperature (after Emerson et al., 1975)

T (ºC)	Ph																
	7.0	7.2	7.4	7.6	7.8	8.0	8.2	8.4	8.6	8.8	9.0	9.2	9.4	9.6	9.8	10.0	10.2
4	0.11	0.18	0.29	0.45	0.72	1.13	1.79	2.80	4.37	6.75	10.30	15.39	22.38	31.36	42.00	53.44	64.53
6	0.13	0.21	0.34	0.53	0.84	1.33	2.10	3.28	5.10	7.85	11.90	17.63	25.33	4.96	46.00	57.45	68.15
8	0.16	0.25	0.40	0.63	0.99	1.56	2.45	3.83	5.93	9.09	13.68	20.08	28.47	38.38	50.00	61.31	71.52
10	0.18	0.29	0.46	0.73	1.16	1.82	2.86	4.45	6.88	10.48	15.65	22.73	31.80	42.49	53.94	64.98	74.63
12	0.22	0.34	0.54	0.86	1.35	2.12	3.32	5.17	7.95	12.04	17.82	25.58	35.26	46.33	57.78	68.44	77.46
14	0.25	0.40	0.63	1.00	1.57	2.47	3.85	5.97	9.14	13.76	20.18	28.61	38.84	50.16	61.47	71.66	80.03
16	0.29	0.46	0.73	1.16	1.82	2.86	4.45	6.88	10.48	15.66	22.73	31.80	42.49	53.94	64.99	74.63	82.34
18	0.34	0.54	0.85	1.34	2.11	3.30	5.14	7.90	11.97	17.73	25.46	35.12	46.18	57.62	68.31	77.35	84.41
20	0.39	0.62	0.98	1.55	2.44	3.81	5.90	9.04	13.61	19.98	28.36	38.55	49.85	61.17	71.40	79.83	86.25
22	0.46	0.82	1.14	1.79	2.81	4.38	6.76	10.31	15.41	22.41	31.40	42.04	53.48	64.56	74.28	82.07	87.88
24	0.52	0.83	1.31	2.06	3.22	5.02	7.72	11.71	17.37	25.00	34.56	45.57	57.02	67.77	76.92	84.08	89.33
26	0.60	0.96	1.50	2.36	3.70	5.74	8.80	13.26	19.50	27.74	37.83	49.09	60.45	70.78	79.33	85.88	90.60
28	0.69	1.10	1.73	2.71	4.23	6.54	9.98	14.95	21.78	30.62	41.16	52.58	63.73	73.58	81.53	87.49	91.73
30	0.80	1.26	1.98	3.10	4.82	7.43	11.29	16.78	24.22	33.62	44.53	55.99	66.85	76.17	83.51	88.92	92.71
32	0.91	1.44	2.26	3.53	5.48	8.42	12.72	18.77	26.80	36.72	47.91	59.31	69.79	78.55	85.30	90.19	93.58

High ammonia values are associated with a deficiency in biological filtration for the simple reason that the bacteria in the filter should consume the ammonia and convert it to nitrite. There is evidence that nitrite can grow a lot (100-700 mg L^{-1}) in the recirculating system with low and almost zero water exchanges. Thus, in the recirculating system the denitrification unit must be cleaned (depending on the load of excrement and food scraps) to discharge the big concentration of nitrite (Van Rijn et al., 2006).

Nitrite concentrations in the recirculating system can be monitored by partially replacing the

water, washing the filters and adding denitrifying bacteria (Camargo et al., 2005; Martins et al., 2010; Schipper et al., 2010; Pulkkinen et al., 2021). There is evidence to suggest that relatively low concentrations of NO_3-N, once thought to be harmless (Wedemeyer & Schild, 1996; Colt, 2006; Lekang, 2013), can influence the toxicity for some of the fish raised in RAS.
The harmful effect on ammonia has negative consequences when the pH rises and a low pH needs very little ammonia to increase the harmfulness (WHO, 1986; Wurts 2003). Elevated ammonia levels in water favor accumulation in the body of ammonia fish that causes death (Randall & Tsui, 2002). Toxic chemicals such as fenix and cyanide ion are becoming more toxic when the pH drops (Könemann, 1986). Hydrocyanic acid is present at low pH, being even more toxic than cyanide ion (Rand, 1995).
In general, research evaluating the chronic toxicity of NO_3-N to crop species at different life stages is limited. Lin & Randall (1990), added caustic soda to the water to raise the pH to 9.91±0.02 for 90 min, and in another tank, they added hydrochloric acid to lower the pH of the tested solution at 3.88±0.02, for 90 min.
At high pH level, the water exhaled by the fish was acidified as it passed through the gills, and at low pH, the exhaled water was alkalinized rather than acidified. Lowering the ammonia in the water could lower the ammonia in the blood. However, blood ammonia increased when the fish were exposed to acidic conditions. Exhaled water pH ranged from 3.88 to 9.91, but exhaled pH only ranged from 4.33 to 7.10, the microenvironment of the fish's fragile gill epithelium managing to maintain a pH balance.
Becke et al. (2017) have studied what happens when rainbow trout is kept in cloudy water, with an increased level of non-ionized ammonia in a RAS over a period of three months and a week. The survived rate was over 99%, without be affected too much of the high turbidity of the water (TSS>25 mg L^{-1}). Ammonia at level of 0.05 mg L^{-1} had no significant negative influence on physiology and trout growth. Also, at load of total suspended solids >10 mg L^{-1}, it was a bacterial growth, which did not affect the fish growth and health.
Davidson et al. (2014) conducted a study on rainbow trout to investigate the effects of nitrate (NO_3) concentrations on fish health. They stocked equal numbers of trout fry in six RAS, maintaining three systems at low NO_3 concentrations (30 mg/L) and three at high NO_3 concentrations (91 mg/L). Trout growth was not affected by mean NO_3 concentrations, but survival was lower where ammonia was added. In addition, lateral swimming and health problems were observed in trout exposed to high concentrations of NO_3.
Anaerobic oxidation of ammonium (anammox process) may be used in recirculating systems as a biological alternative for ammonia removal (Van Rijn et al., 2006). This oxidation of ammonium is very important for RAS. Oxidation allows complete removal of autotroph nitrogen, unlike the traditional mode with nitrification biofilters and heterotrophic denitrification systems that require the addition of organic carbon. Therefore, the oxidation process uses half of the ammonia that fish produce and the rest is converted anaerobically into nitrogen gas along with the produced nitrite. Anaerobic oxidation can reduce the consumption of electricity and the excessive use of dissolved oxygen (Van Rijn et al., 2006).

Carbon Dioxide

Carbon dioxide (CO_2) is toxic to fish and has a limiting effect on RAS. The accumulation of CO_2 is favored by the high density of fish. A high level of CO_2 in water leads to acidification of the blood and reduced oxygen absorption (Molleda, 2007).
At lower alkalinity levels (10 mg L^{-1}), CO_2 removal is significantly higher compared to higher alkalinity (70 and 200 mg L^{-1} $CaCO_3$). When assessing total inorganic carbon loss from the RAS, daily loss was found to be roughly equal at 10 and 70 mg L^{-1}, but highest at 200 mg L^{-1} alkalinity. Additionally, pH records indicated that the 10 mg L^{-1} alkalinity treatment led to the lowest system pH (Summerfelt et al., 2014).
At trout, the excretion of CO_2 is about 1-2 mg CO_2 kg^{-1} min^{-1}, and it can bear a maximum 20 mg CO_2 L^{-1} in water (Timmons et al., 2018). Elimination of carbon dioxide occurs with the

help of compliant (gassing tunnels) (Summerfelt et al., 2003); by injecting very small air bubbles that favor atmospheric air intake and CO_2 removal (Barrut et al., 2012); air transport pump with CO_2 removal that can remove 13-20 g CO_2 kW h^{-1} (Loyless & Malone, 1998); aeration devices that can remove 1.2 kg CO_2 kW h^{-1} (Eshchar et al., 2003).

Photoperiod

Ma, et al. (2023) have researched how different types of lights influence the juvenile rainbow trout. Spectra of blue-violet-red light, blue-violet red light, blue light, and red light alternated at 300, 900, and 1200 lx. At the end of the research, they noticed better results with the help of red violet blue light that showed a significant increase in lipase, a better digestion process in the stomach, and increased feed consumption.

Red light has been suggested for intensive rearing of rainbow trout also by Karakatsouli et al. (2008). Good results can be achieved with spectra of light trying to copy light from nature.

Salinity

Some fish like rainbow trout have a higher salt tolerance, which gives them an advantage for growth in RAS (Tian et al., 2022).

Bordignon et al. (2024) wanted to see if three levels of water salinity in an aquaponic system (low: 0.5‰; average: 3.0‰; high: 6.0‰) may influence the growth performance and characteristics of the trout housing. The addition of salt to the water did not affect the weight and the trout filet, also salmastra water with up to 6‰ reduced the consumption of freshwater and did not affect the growth of fish and leafy vegetables or the overall balance of the aquaponic system. After 21 days, considered adaptation period, salt was added little by little to 3‰ in systems with average salinity and 6‰ in high salinity systems observing a slight salinity effect water on microbial communities in water, namely the measured bacterial ecosystem diversity, has decreased to salinity of 6‰, observed by other researchers in other saline environments such as mud (Ya el al., 2023), and coastal lakes (Lew et al., 2022). Microbial analysis has shown that there are no significant changes between salinity doses used.

Alkalinity and Hardness of Water

Reaction between different types of alkali ions such as: hydroxide; carbonate; hydrogen carbonate and water pH can cause total alkalinity (Boyd et al., 2016; Lindholm-Lehto, 2023).

Alkali ions HCO_3^-, CO_2^- and OH^- are found naturally in water (Boyd et al., 2016). Due to the calcium and magnesium salts present in the water alkalinity is measured in mg $CaCO_3$ L^{-1}. Due to the limestone present in the water, it may have a certain hardness, the water hardness is measured in mg L^{-1} or German degrees. Sweet water hardness is considered between 5 mg L^{-1} and over 500 mg L^{-1} (Timmons et al., 2018).

The addition of acidic or basic solutions in RAS water can change the level of alkalinity or acidity of the pH in the water (Boyd et al., 2016). Also, temperature can influence together with alkalinity and pH carbon dioxide dissolved in water (Timmons et al., 2018).

Chen et al. (2006), consider that an alkalinity value of 200 mg $CaCO_3$ L^{-1} is very good for the nitrification unit, but Timmons et al. (2018), found it is better to use a value between 50-300 mg $CaCO_3$ L^{-1}.

The energy consumed for osmoregulation is lower in hard water than fine (Klontz, 1991).

Sudden and extreme changes in water hardness, even in combination with increased water temperature, do not cause trout mortality (Huysman et al., 2022).

Turbidity

Total suspended solids (TSS) directly influence water turbidity, which can impede fish vision and ultimately compromise their life cycle. At 160 nephelometric turbidity units (NTU), i.e. approximately 54.4 mg L^{-1} (TSS), trout feeding is not affected (Greer et al., 2015).

In a RAS, about 25% of the water turbidity comes from fish feed (Cripps & Bergheim, 2000).

Suspended solid particles can cause stress reactions and endanger the health of aquatic animals (Alabaster & Lloyd, 2013). An increase in concentration (TSS) can lead to increased oxygen consumption, and poor biofilter performance (Michaud et al., 2006).

CONCLUSIONS

The optimum temperature for growing rainbow trout can fluctuate between 12°C and 17°C, and higher or lower temperatures have adverse effects influencing growth, viability, appetite, digestive capacity and antioxidant capacity.

Dissolved oxygen can improve the health of fish, but it cannot eliminate the causes.

Using ozone with caution helps maintaining water parameters for fish.

Improper use of ozone can cause high mortality among fish.

Ultraviolet light kills most parasites and bacteria and should be installed after the biological filter of the recirculating system. Heavy metals can influence the pH, and its decrease raises a question mark about their existence in the water.

Water pH in recirculating trout rearing systems is crucial to trout health and rearing performance. An optimum pH for trout in such systems is generally between 6 and 9. Failure to meet these pH limits can affect nutrient uptake, metabolism and fish stress. Regular pH monitoring and adjustment are essential to maintaining an optimal balance in recirculating trout rearing systems. Higher than normal pH values can cause high mortalities among fish, especially in the initial stages of development. Biological effects of high or low pH can affect the development of fish. Following certain symptoms of fish, including eye exophthalmos, skin or gill injuries, can suspect an unfavorable pH that can even cause death.

pH can influence the biology and chemistry of water, so it is important to carefully observe the complex interactions between pH, dissolved oxygen, ionic strength and ammonia concentration in the damaged environment

In the aquatic environment is an important complex interaction between pH, dissolved oxygen, ionic strength and ammonia concentration.

Ammonia (NH_3) is harmful to trout, but ammonium ion (NH_4) is practically harmless.

High concentrations of nitrite are harmful to fish life and can increase mortality.

Carbon dioxide is a limiting factor in recirculating systems and the maximum concentration allowed for rainbow trout is 20 mg L^{-1}.

Artificial light spectrums that mimic natural light are beneficial and can be used in recirculating systems. Red and blue light can ensure a well-being of fish.

The alkalinity can fluctuate around the value of 50-300 mg of $CaCO_3$/L, and at a value above 6‰ the salinity destroys the balance of the nitrifying and denitrifying bacteria in the biological filter.

Turbidity is closely related to the size of the space, the density of the fish, the food and the efficiency of the filtering biosystem, and it is preferable that the water be as clean as possible.

The subject being so vast and important for the growth of trout in recirculating systems, certainly the research in the field will expand, and the discovery of new technologies will lead to the adjustment of the limiting parameters that will ensure an optimal aquatic environment.

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EXPLORING UNCONVENTIONAL PLANT-BASED INGREDIENTS AND THEIR INFLUENCE ON SUSTAINABLE TROUT FARMING PRACTICES

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Abstract

Although fishmeal is an excellent feed component for trout, there are concerns about its sustainability. Overfishing and the environmental impact of fish meal production have raised questions about its long-term availability. Consequently, there's a growing interest in finding alternative protein sources for fish feed, such as plant-based proteins. The exploration of non-conventional ingredients in trout feed stems from a pressing need to safeguard the environment, promote aquaculture sustainability, and enhance consumer safety. By ensuring the safety of food products, consumer trust in purchasing aquaculture-derived fish is likely to increase, potentially reducing reliance on wild-caught fish. Within this framework, a comprehensive literature review was conducted to identify novel ingredient sources suitable for trout feed. This involved examining the origins of new plant-based ingredients, assessing their physical, chemical, and microbiological characteristics, and evaluating their impact on trout feed production. The findings of this study can inform and enhance feed management strategies in aquaculture, aligning with sustainability objectives and efforts to boost productivity.

Key words: *aquaculture, alternative fish diets, go green, emerging feed resource, environmental issues.*

INTRODUCTION

To ensure the sustainability of animal and its long-term viability, it is important to discover novel feed resources with superior nutritional value and conversion efficiency. Simultaneously, it is important to enhance the quality of animal products and maximize the efficient utilization of land and water resources (Holman & Malau-Aduli, 2012).

In recent years, the fish industry emerged as one of the fastest-growing sectors in global food production. In 2022, global fisheries and aquaculture production hit an unprecedented high of 223.2 million tons, comprising 185.4 million tons of aquatic animals and 37.8 million tons of algae. Of the total aquatic animal production, 62 percent was sourced from marine areas (with 69 percent from capture fisheries and 31 percent from aquaculture), while 38 percent was from inland waters (84 percent from aquaculture and 16 percent from capture fisheries) (FAO, 2024). This expansion has resulted in an increasing demand for fishmeal, an essential ingredient widely used in aquaculture feeds (Habib et al., 2008; Macusi et al., 2023; Rosenau et al., 2023). Therefore, aquaculture has outpaced other sectors and stands as the main consumer of fishmeal (Bachis, 2022). Following declining catch rates of wild fish, environmental issues associated with fishing methods, and the excessive energy and water requirements in the production of aquatic feed, finding of sustainable alternatives for fish fodder has become necessary (Holman & Malau-Aduli, 2012; Zhang et al., 2020; Rosenau et al., 2023). According this target, the World Trade Organization, in 2022, in Geneva, obtained a historic agreement between 165 countries aimed at reducing fishing subsidies and reducing overfishing worldwide.

Aquaculture confronts a significant challenge due to the increasing worldwide need for seafood coupled with stagnation in fishery capture production (Holman & Malau-Aduli, 2012). Aquaculture production had exceeded capture fisheries production for all sectors except marine finfish, which remains dominated by capture fisheries (Mair et al., 2023). 89% of the total aquatic animal production was intended for human consumption, providing an average of 20.7 kg per person. The per capita apparent consumption of aquatic animal foods has steadily increased, rising from 9.1 kg in 1961 to 20.6 kg in 2021, at an average annual growth rate of 1.4 percent. This growth has been fuelled by increased supply, advancements in preservation and distribution technologies, evolving consumer preferences, and rising incomes (FAO, 2024).

The reliance of aquaculture on feed ingredient produced by cooking, pressing, drying, and grinding fish or fish waste, which is widely condemned for the negative ecological effect, presents a sustainability concern. To ensure the sustainable expansion of aquaculture, it is essential to integrate alternative protein and lipid sources. Given the rapidly rising prices of food sources that provide nutrients for fish, as well as increasing demand of sustainable fisheries, a pressing necessity emerge to identify substitutes to these ingredients in compound feeds used in aquaculture (Zhang et al., 2020).

Therefore, possible protein and lipid resources which can serve as alternatives for fish meal and oil are being sought in plants. These substitutions have shown promise, given their biochemical composition and bioavailability. Nevertheless, usage of plant-based oil does come with certain disadvantages, primarily related to adulteration of fat structure in the fish's muscular tissue, and secondly their extractability (Bell et al., 2001; Torstensen et al., 2005; Pettersson et al., 2009).

One of the most traded fish species is rainbow trout *Oncorhynchus mykiss*. Currently, trout farming is spread all over the world: North and South America, Oceania, Europe, Asia, and Africa. In 2020, the worldwide production of rainbow trout reached approximately 960 K tons, representing approximately 4% from the total amount of farmed fish (Rashidian et al., 2020; Vaclavik et al., 2020; FAO, 2022).

In the context of Blue Transformation and in alignment with Go Green principles, sustainable aquaculture can address some of today's most urgent challenges. This includes ensuring food security, reducing the strain on wild fish populations, and lowering the climate and environmental impact of our food system. Additionally, it can provide consumers a wider variety of healthy and sustainable food products.

The review presents a bibliographic analysis focused on exploring new alternative vegetable ingredients and protein sources for trout feed, aiming to address concerns about the sustainability of fishmeal.

The study aims to support several significant practical point of view:

1. Reducing dependence on animal protein. Using plants in trout feed can reduce the need for animal protein, which is often obtained from sources such as small wild-caught fish. This can contribute to the conservation of natural resources and the protection of marine ecosystems.
2. Increasing food sustainability. Partial or total replacement of conventional trout feed ingredients with plants can contribute to greater sustainability of the food chain. Growing plants can be less intensive and less polluting than raising animals for food.
3. Improve the nutritional quality of feed. Choosing plant-based ingredients can improve the nutritional profile of trout feed by providing beneficial substances such as antioxidants, essential fatty acids and vitamins that can support fish health and well-being.
4. Reducing Ecological Footprint. Growing plants for food is often less harmful to the environment than raising animals. Reducing the ecological footprint of aquaculture through the use of plants can help maintain the health of aquatic ecosystems and conserve biodiversity.
5. Promoting innovation and research in the aquaculture industry. The study and implementation of non-conventional plant-based ingredients in trout feed can stimulate innovation in the aquaculture industry. This can open up new opportunities for research and development of more sustainable and healthier food products.

This study not only brings practical benefits, but can facilitate the transition to a more ecological and sustainable aquaculture model, contributing to protecting the environment and ensuring a sustainable food resource for the future.

MATERIALS AND METHODS

The review conducts an extensive examination of prior research concerning alternative feed constituents for trout, aiming to establish a comprehensive foundation for future investigations and experiments focused on substituting conventional feed components with non-traditional plant-based alternatives.

The study catalogued and analysed these alternative ingredients, mapping their geographic origins and conducting detailed assessments of their physical, chemical, and microbiological attributes.

Moreover, the study scrutinized the production implications associated with integrating these alternative plant-based constituents into trout feed formulations.

It explored the nuanced advantages and potential disadvantages of various unconventional feed sources, underscoring the urgent necessity for expanded research and development efforts in this field. The overarching goal is to ensure the sustainability and responsible advancement of aquaculture practices.

The research methodology involved leveraging a range of intensive research databases, including Web of Science, Google Scholar, PubMed, Scopus, and Science Direct.

These platforms were pivotal in gathering a comprehensive array of relevant papers covering diverse aspects of the environmental, economic, and nutritional impacts associated with alternative feed ingredients for trout.

Additionally, to enrich the literature search process, consultations were conducted with fisheries and aquaculture scientists, providing valuable insights and expert perspectives.

This holistic approach not only consolidates existing knowledge but also lays the groundwork for future studies aimed at optimizing feed formulations for trout farming.

By critically evaluating and integrating findings from multiple sources, this paper contributes significantly to the ongoing discourse on enhancing the sustainability and efficiency of aquaculture practices worldwide.

RESULTS AND DISCUSSIONS

New "green" ingredients

Developing plant-based ingredients for trout feed is a dynamic and expanding area of research and innovation within aquaculture. These ingredients are increasingly sought after due to their potential to mitigate the reliance on traditional marine-based components such as fishmeal and fish oil. The overuse of these marine resources has raised significant concerns about overfishing, which threatens marine biodiversity and disrupts ecological balance.

Plant-based ingredients offer a sustainable alternative that can help preserve marine ecosystems. They include a variety of sources such as microalgae and seeds from various plant species, each providing unique nutritional benefits. These ingredients are rich in essential nutrients like proteins, amino acids, vitamins, and fatty acids necessary for the healthy growth and development of trout.

Moreover, the use of plant-based ingredients can contribute to reducing the carbon footprint of aquaculture operations. Plant cultivation typically results in lower greenhouse gas emissions compared to fishing and fish processing. Additionally, the shift towards plant-based feeds can spur agricultural innovation, leading to more sustainable farming practices and the development of crops specifically tailored for aquaculture feed.

Incorporating plant-based ingredients into trout feed also addresses consumer demand for more sustainable and ethically produced seafood. With growing awareness about environmental issues and sustainability, consumers are increasingly favouring products that are not only healthy but also environmentally responsible. Aquaculture producers who adopt plant-based feed ingredients can tap into this market trend, enhancing their brand image and marketability.

Furthermore, ongoing research is focused on overcoming challenges associated with plant-based feeds, such as digestibility and palatability for trout. Advances in food technology and nutritional science are leading to the development of feed formulations that optimize the benefits of plant-based ingredients while ensuring the health and growth performance of the fish (Ivan et al., 2022).

The development of plant-based ingredients for trout feed is a key for the sustainability and future of aquaculture. It helps reduce environmental impact, supports marine conservation, meets consumer demands for sustainable products, and drives agricultural and nutritional innovation.

The main vegetable sources used in trout feed are algae (*Spirulina* sp., *Chlorella vulgaris, Tisochrysis lutea, Tetraselmis suecica, Nannochloropsis* sp.) and seeds rich in oils and protein from the plant species of the Brassicaceae Family (*Brassica napus* - rape, *Brassica carinata* - ethiopian mustard, *Camelina sativa* - false flax), sunflower (*Helianthus annuus*), lin (*Linum usitatissimum*), coconut (*Cocos nucifera*), and cotton (*Gossypium arboreum*).

It is important to note that the formulation of trout feed with plant-based ingredients should be carefully balanced to meet the nutritional requirements of trout. Additionally, ongoing research is essential to determine the optimal combination of plant-based ingredients and to assess their impact on trout growth, health, and product quality. Researchers and aquaculture companies are continually working to develop and optimize plant-based trout feed formulations to make trout farming more sustainable and environmentally friendly.

Areas of origin for vegetal ingredients

Several nations produce vegetable ingredients for fish feed. To comprehend the production of diverse plant-based components in fish farming and aquaculture, a distribution map was crafted. It delineates the principal countries engaged in production, organized in descending order according to productivity. The data utilized is derived from FAO statistics for the year 2022 (Figure 1) (FAO, 2023).

Figure 1. Different ingredients production used for Fishery and Aquaculture (original, using Google Maps) (https://www.google.com/maps/d/edit?mid=1NSaiLeO-DQkj6LQwxIAZBBVtCmv8CTk&usp=sharing)

Algae are considered a greatly appreciated type of protein, as well as essential amino acids (Fabregas et al., 1985; Becker, 1993) and vitamins (Becker, 2004). Global algae production (including cultivation and wild collection) increased over 3 times from 11.8 million (wet) tons in 2000 to 35.82 million tons in 2019; nearly all the growth was contributed

by cultivation, while wild collection was relatively constant (Figure 2).
In 2022, the global algae production surged to 37.8 million tons (wet weight) from aquaculture, and 1.3 million from wild collection (FAO, 2024).

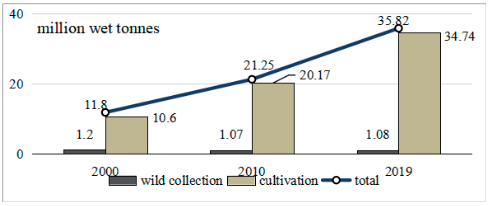

Figure 2. Global algae production, 2000-2019 period (based on data sourced from FAO, 2021)

In 2019, 35.8 million tons of world algae (including seaweeds and microalgae) production were contributed by 54 countries/territories with 97 percent of the production coming from cultivation. From total world production of algae (including seaweeds and microalgae of aquaculture and wild collection), the highest percentage from the total world production and the use of this production in aquaculture is in Asia (FAO, 2021) (Table 1).

Table 1. Global algae (including seaweeds and microalgae) production, in 2019 (based on data sourced from FAO, 2021)

Area/ Country	Total production (tons)	Share of world total (%)	Aquaculture share in total production (%)
World	35 818 961	100.00	96.98
Asia	34 881 600	97.38	99.10
China	20 351 442	56.82	99.14
Indonesia	9 962 900	27.81	99.55
Japan	412 300	1.15	83.80
Americas	488 144	1.36	4.87
Chile	427 508	1.19	5.28
Peru	36 348	0.10	0.00
Canada	12 655	0.04	0.00
Europe	287 386	0.80	3.99
France	51 683	0.14	0.74
Norway	163 197	0.46	0.07
Russia	19 544	0.05	54.10
Africa	145 259	0.41	81.33
Tanzania	106 069	0.30	100.00

Arthrospira is a type of cyanobacteria that forms tubular, multicellular trichomes in an exposed leftward spiral. Two species, *A. platensis* and *A. maxima*, are used to produce a dietary supplement known as spirulina. Large-scale microalgae culture began in Japan in the 1960s with *Chlorella*, and then Spirulina in 1970 in Mexic. Spirulina now is cultivated in over 20 states, including Brazil, China, India, Peru, Spain, and the U.S.A. The amount of spirulina produced worldwide on an annual basis is estimated to be around 3,000 tons per year (Shimamatsu, 2004), but FAO FishStat data suggests that the industrial production of spirulina is much higher than the previous estimate of 3,000 tons per year (FAO, 2006).
Besides the nations reported in FishStat, there are other significant producers of spirulina such as the U.S.A. (3394 tons in 2019), Taiwan, and Thailand, which are not accounted for in the FishStat reports. Spirulina can serve as a cost-effective alternative to animal-based protein sources in aqua feeds, and can either partially supplement or completely replace them. Compared to animal-derived feed ingredients, spirulina is a relatively low-cost option.
Rape (*Brassica napus*), originated as a cultigen in Southern Europe, it is now a domesticated plant species grown on almost all continents. The cultivation of *B. napus* started 6000 years ago in India, and it subsequently extent to East Asia around 1st century AD (Snowdon et al., 2007). Currently, *B. napus* is ranked as the third major variety of plant oil globally, just behind soybean and palm oil. Additionally, it is also the second main protein alternative globally, following soy (Heuzé et al., 2020). In 2021, world rapeseed production was 73.95 million tons, from which 26.58 million tons were canola oil. Normally, the European Union is responsible for about a third of the world's rapeseed production, which amounts to 60 million tons annually (FAO, 2023).

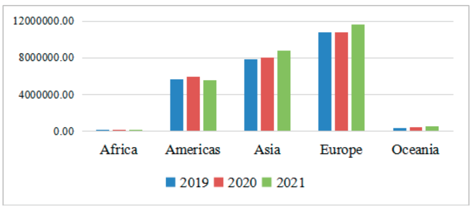

Figure 3. Production quantities of Rapeseed oil, crude (based on processed data from FAO, 2021)

In the European Union, Germany emerged as the leading producer with 3.505 million tons in

2021, contributing significantly to the total production. Beyond the EU borders, major producers include Canada, China, India, Australia, Russia, Ukraine, and the USA, while Africa and Oceania account for the lowest production levels (Figure 3) (FAO, 2023).

Ethiopian mustard (*Brassica carinata*), is a crop that is cultivated for its oilseed in Ethiopia. The culture of this plant was also introduced in other African countries, such as: Gabon, Ivory Coast, Kenya, Tanzania, and Uganda.

Lin or **flax** (*Linum usitatissimum*) is known as a plant rich in nutritional ingredients. It has been cultivated for thousands of years for germs, which is eaten whole, crushed, or pressed to produce flaxseed oil. Flax is taken into account for its possible welfares characteristics, for fuel or as fiber crop. The fat from seeds is so-called linseed oil and can be consumed as animal forage.

Sunflower (*Helianthus annuus*), which accounts for 8% of global oilseed production, is the fifth most cultivated oilseed crop worldwide. Originally native to Central North America, it has been widely distributed across the globe and is now found in regions ranging from Russia to South America.

In the realm of flaxseed productions obtained, Europe claims the top position, averaging 96.4% from 2019 to 2021. Oceania registers at 0%, while Asia, Africa, and the Americas collectively reach percentages of 3-4%. The largest producer in Europe is France with a production of 757680 tons. Also, the largest production of sunflowers worldwide is obtained in Europe (75%), and the largest producer in Europe is Romania (2,845,183.33 tonnes). More than 50% of the world's sunflower seed production is contributed by Ukraine, Russia and Argentina combined (FAO, 2023).

Coconut (*Cocos nucifera*), belongs to the *Arecaceae* Family and is the only surviving species in the Cocos genus. It is primarily found in the wet tropical biome and is omnipresent in warm areas by the seaside. It is considered a traditional image of the tropics. *C. nucifera* native range extends from Central Malesia to SE. Pacific and was originally farm by the indigenous ethnic minorities who lives on the islands in the Southeast Pacific and Indian oceans. The coconut tree was spread during the Neolithic period through seaborne migrations. The coconut tree was brought to various regions including India, Southeast Asia, Central Africa, Madagascar, and parts of North and Central America and South America. Majority of the worldwide coconut resource is now dominated by three countries, with the Philippines producing 42%, Indonesia producing 25%, and India producing 12%. In coconut oil production, Asia dominates with a productivity share ranging from 86% to 88% of the global production (Figure 4). The coconut tree is a versatile plant that has various uses: source of fuel, food and animal feed, medicinal plant and toxic substance.

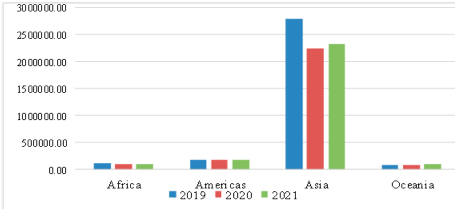

Figure 4. Production quantities of coconut oil (based on processed data from FAO, 2021)

Wild cotton species (*Gossypium* Genus) have been found in Mexico, Australia, and Africa, among other regions. Cotton plant was first domesticated in the Old World (likely in the Indus Valley of modern-day Pakistan) around 4500 BCE, and then independently in the New World (likely in present-day Peru) around 3600 BCE. There are about 50 Gossypium species and *Gossypium hirsutum* is the highly grown kind of cotton plant, representing almost the entire cotton crop in the world. *Gossypium barbadense* is the second most commonly grown species, representing around 2-3% of cotton global production. The remaining 1% is made up of *Gossypium arboreum* and *Gossypium herbaceum*, which are less widely cultivated (Chaudhry, 2010). Cotton is one of the primary natural fibers used by humans today, comprising approximately 80% of world natural fiber production. It is a major oilseed crop and a significant source of protein for animal feed where it is cultivated. Cotton is very important for agriculture, industry, and trade, particularly for tropical and subtropical countries in Africa, South America, and Asia.

As a result, the *Gossypium* genus has been a subject of interest for scientists for a long time.
The oil obtained from cotton seeds were a dietary staple in U.S.A. in the last hundred years, and prior to 1940s, it was the primary plant oil manufactured in this country. Currently, it is the third oil produced from plants in U.S.A., with a yearly average quantity of over 500,000 tons. *Gossypium* sp. oil represents up near 5-6% from whole home fat and oil source and is one of the most unsaturated oils, along with oils from seed plants as *Carthamus tinctorius, Zea mays, Glycine max*, *Brassica campestris*, and *Helianthus annuus*.
The largest cotton-producing region is Asia, followed by the Americas and Africa, Europe and Oceania registering the lowest productions (Figure 5) (FAO, 2023).

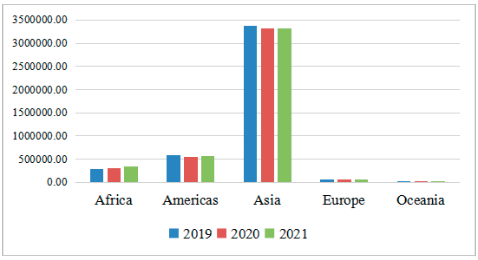

Figure 5. Production quantities of cotton oil (based on processed data from FAO, 2021)

Physical, chemical and microbiological characteristics of some plant-based ingredients

There are several species of **spirulina**, but the most commonly safe considered for consumption are *Arthrospira platensis* and *Arthrospira maxima*. Other species of spirulina include *Arthrospira fusiformis* and *Arthrospira biomass*, but these species are less commonly used for consumption. *Arthrospira* sp. is known for its high protein content. It typically contains around 50-70% protein by dry weight, more than other commonly used plant sources, which is way it is considered one of the most protein-rich food sources available (Phang et al., 2000). *Arthrospira* sp. is known to contain all essential amino acids, in a balanced composition, making it a complete protein source. Additionally, it contains high levels of fat molecule that contains two or more double bonds in their carbon chain (PUFAs) such as gamma-LA (GLA), which is a ω-6 fatty acid. *Arthrospira* sp. is also an abundant in vitamins, minerals, and photosynthetic pigments (Spolaore et al., 2006; Habib et al., 2008).
Spirulina is easily digestible due to the lack of cellulose in its cell walls, which is a unique benefit. Spirulina powder is a low-fat, low-calorie, and cholesterol-free source of protein, typically containing 60% protein, 20% carbohydrate, 5% fats, 7% minerals, and 3-6% moisture, making it nutritionally balanced.
Spirulina is a source of protein, with a high concentration fluctuating from 55% to 70% by dry weight, which exceeds most other commonly used plant sources. Its protein is complete and contains all essential amino acids, although with moderated levels of methionine, cysteine, and lysine equated with ordinary proteins found in products of animal origin. However, spirulina's protein profile is superior to other plant-based proteins such as those found in legumes. Spirulina is highly digestible because of the lack of cellulose in its cell walls, making it easy to assimilate.
Spirulina is rich in polyunsaturated fatty acids (PUFAs), comprising 1.5-2.0% of its total lipid content of 5-6%. Notably, spirulina is a good source of γ-linolenic acid (30-35% of total PUFAs), and also contains alpha-linolenic acid (ALA), linoleic acid (LA, 36% of total), stearidonic acid (SDA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic acid (AA). Spirulina platensis is a promising source of γ-linolenic acid, which can be further increased by growing it under light-dark cycles either in a test centre or outside (Tanticharoen et al., 1994). Spirulina is abundant in a variety of minerals (potassium, calcium, chromium, copper, iron, magnesium, manganese, phosphorus, selenium, sodium, and zinc) and vitamins like thiamine (B1), riboflavin (B2), nicotinamide (B3), pyridoxine (B6), folic acid (B9), cyanocobalamin (B12), vitamin C, vitamin D, and vitamin E. Additionally, it contains high levels of β-carotene, which can be transformed to vitamin A. The presence of these vitamins, along with iron, potassium, and chlorophyll, can help promote the metabolism of carbohydrates, fats, and proteins, as well as support the growth and reproduction of skin, muscle, and mucosa.

Spirulina possesses the remarkable ability to detoxify and chelate toxic minerals. This means that it can neutralize or remove harmful substances from water and food, such as arsenic. Additionally, it has the potential to chelate or eliminate the poisonous effects of heavy metals found in water, food, and the environment.

Spirulina is rich in various pigments, including chlorophylla, xanthophyll, betacarotene, echinenone, myxoxanthophyll, zeaxanthin, canthaxanthin, diatoxanthin, 3-hydroxyechinenone, beta-cryptoxanthin, oscillaxanthin, as well as the phycobiliproteins c-phycocyanin and allophycocyanin.

The biological and chemical configuration of spirulina has been extensively studied, with analyses conducted on spirulina grown in different conditions, including laboratory settings, natural environments, and mass culture systems that use agroindustrial waste effluent. The composition of spirulina was set up to differ in feedback to the salt percentage of the growth environment. Additionally, detailed biochemical analyses have revealed the presence of various compounds and nutrients in spirulina, including protein, essential fatty acids, vitamins, and minerals. Vonshak et al. (1996), reported that the biochemical composition of salt-adapted cells differs from non-adapted cells, as they exhibit a decline in protein and chlorophyll and an upsurge in carbohydrate content.

Commercial production of spirulina is primarily based on superficial raceways where spirulina are agitated by a rotary vane. Nevertheless, there are still some instances where spirulina is commercially collected from natural environment.

When spirulina is cultivated in a laboratory setting, its productivity can be influenced by eight major environmental factors. These include luminosity (with a photo-period of 12/12 and 4 lumens), temperature (30°C), inoculation size, mixing rate, melted solids (ranging from 10 to 60 g/litre), pH (8.5–10.5), aquatic feature, and the existence of macro and micronutrients such as carbon, nitrogen, phosphorus, potassium, sulfur, magnesium, sodium, chlorine, calcium, iron, zinc, copper, nickel, cobalt, and selenium (Ciferri, 1983). Spirulina is a highly nutritious and sustainable food source that can be produced on a small scale with minimal resources, making it an attractive option for communities in need of nutritional support and economic development. Additionally, spirulina cultivation has the potential to mitigate environmental problems such as nutrient pollution and greenhouse gas emissions. Overall, the cultivation and consumption of spirulina has the potential to improve human health and well-being while promoting environmental sustainability (Habib et al., 2008).

Plant species of the Brassicaceae Family, like rape, have a primary purpose of cultivation for the oil-rich seed, which contains a significant amount of erucic acid (up to 50%) and glucosinolates. Erucic acid is known to cause damage to the heart muscles of animals, while glucosinolates have been found to interfere with iodine metabolism, leading to physiological disorders in the liver, kidneys, and thyroid glands. This interference can reduce growth and overall performance. The oils from the seeds of these species (camelina) are used in human and animal feed, as biodiesel for diesel engines and even as an aviation biofuel for jet aircraft engines (Resurreccion et al., 2021).

Rapeseed meal, or canola meal, is a by-product of the extraction of rapeseed oil from the seeds of *Brassica* species such as *Brassica napus, Brassica rapa, Brassica juncea*, and their hybrids. This residue is rich in protein and widely used as animal feed for various livestock. It is the second most commonly produced meal globally, after soybean meal (Heuzé, 2020). As fish meal supplies decline, vegetable-based proteins are being sought after by the aquaculture industry to provide the necessary amino acids for the high protein necessities of many farmed fish species, which are largely carnivorous. Canola meal has emerged as a significant ingredient in aquaculture diets across the globe, as it is able to fulfill this demand for vegetable-based protein. Although certain obstacles persist, several observations have demonstrated that canola meal can be an effective component in many fish food (Feed Industry Guide, 2019).

Ethiopian mustard seed contains great ranks of glucosinolates and erucic acid, which are considered undesirable for human and animal consumption. As a result, *B. napus* is preferred as an oilseed crop. However, the secondary product of *B. carinata* oil fabrication is consumed as protein meal as farm feed.

Linseeds and **linseed meal** gained significant interest after 1990 because of their great percentage of polyunsaturated fatty acids (PUFA), mainly alpha-linolenic acid (ALA) and conjugated linoleic acid (CLA). ALA is an omega-3 fatty acid, and linseeds and linseed meal are particularly rich in it (54% of the fatty acids). In addition to ALA, linseeds and linseed meal contain c18:1 (19%) and c18:2 (15%) fatty acids. Providing these fatty acids to livestock intakes it is changing the fatty acid balance of animal products in order to make more available one for consumers and for people wellbeing (Heuzé et al., 2018).

The great content of omega-3 fatty acids (ALA) in linseed meal causes a higher level of unsaturated fat in animal products, which leads to a smaller storing period. Additionally, the high omega-3 composition poses another difficulty since this fatty acid oxidizes quickly and becomes rotten, further reducing the storing interval.

Australia has developed cultivated varieties of *L. usitatissimum* that are specifically farmed to produce linseed oil with a reduced alpha-linolenic acid content. Linola was introduced in the 1990s as a low-linolenic acid variety of linseed, with reduced levels of omega-3 fatty acids. This made it a better option as fodder, as the high omega-3 content of linseed meal can cause a higher unsaturated fat content in animal products and shorten their storage time. However, linseeds and linseed meal still have some disadvantages. They contain a vitamin B6 (pyridoxine) antagonist, which may need additional supplementation. Additionally, linseeds contain 2-7% of mucilage (fibre), which are not processed by monogastric animals and may be harmful to young animals without treatments with enzymes (Heuzé et al., 2018).

Linseed meal is possible to be a potential protein supply in fish foods, but it has certain limitations because of its amino acid imbalance and antinutritional elements such as mucilages, tannins, phytates, and HCN. Feeding linseed meal in fish forages is restricted because of these factors, and protein digestibility which is generally low-slung, for instance, 70% in rainbow trout. However, these issues can be addressed through demucilagination, fermentation, and amino acid supplementation. By treating linseed meal, it can be administered to substitute 25 to 75% of the intake in fish feeds.

In rainbow trout, the digestibility of energy and protein from linseed meal is generally lower compared to other oil meals, with an average of 34% for energy and 70% for protein. In comparison, soybean meal has higher digestibility values of 77% and 89%, respectively, for energy and protein. Additionally, linseed meal has lower availability of essential amino acids such as histidine, valine, isoleucine, and lysine, which can limit its use as a protein source in fish diets (Gaylord et al., 2008; Gaylord et al., 2010).

Sunflower seeds. The original sunflower oil, also known as linoleic sunflower oil, has a high content of polyunsaturated fatty acids, particularly linoleic acid, which makes up about 68% of its fatty acid profile. It is also low in saturated fats, such as palmitic and stearic acid. Nevertheless, new hybrid varieties of sunflowers have been bred to adjust the fatty acid outline for specific purposes, such as high oleic sunflower oil, which has an upper concentration of monounsaturated fats and poorer values of polyunsaturated fats, making it more stable for high-heat cooking applications.

Sunflower seeds are grown primarily for their oil, but the by-product of oil extraction, sunflower meal, is also an appreciated component in livestock feeds due to its high protein content. Sunflower meal is commonly used in the diets of poultry, swine, and dairy and beef cattle as a source of essential amino acids and protein. Sunflower protein contains less lysine (around 4% protein) compared to soybean protein but has relatively higher levels of sulfur-containing amino acids, such as cystine and methionine (1.9% and 2.2% protein, respectively). Unlike other major oilseeds like soybeans, cottonseeds, and rapeseeds, sunflower seeds do not contain antinutritional factors, making it a safe feed for all livestock species. However, there may be concerns about residues and contamination (such as pesticides, insecticides, and mycotoxins like aflatoxin B1 and ochratoxin) during sunflower cultivation, harvest, and post-harvest operations (Heuzé et al., 2015).

Coconut oil has a natural flavour and aroma characteristic of coconut, and contains only a minor quantity of unsaponifiable substance along with trace amounts of tocopherols, tocotrienols, and phytosterols. It is colourless and composed of 92% saturated fatty acids, primarily triglycerides. Around 8% of the fatty acids are monounsaturated and polyunsaturated fatty acid. Most of the saturated fatty acids in coconut oil (nearly 70 percent) are medium-chain fatty acids (MCFAs). Medium chain fatty acids (MCFAs) are not commonly found in other plant oils, and coconut oil is unique in that it contains a high proportion of lauric acid (C12:0), ranging from 50-60%. Different portions of coconut oil also contain medium chain triglycerides. Because of its high MCFA content, the metabolism of coconut oil is different from that of other vegetable oils that mainly contain long chain fatty acids. Therefore, it cannot be assumed that coconut oil has properties similar to those of oils or fats that are primarily composed of long chain saturated fatty acids (92%). Coconut oil is reputed to have antibiotics effects, as well as exceptional medicinal qualities (Gopala et al., 2010; Perera, 2016).

Cottonseed oil is a type of herb oil that has an upper quantity of polyunsaturated fatty acids associated to saturated fatty acids, with a ratio of 2:1. The majority of its fatty acid profile is made up of 70% unsaturated fatty acids, which includes 18% monounsaturated (such as oleic acid), 52% polyunsaturated (such as linoleic acid), and 26% saturated fatty acids (primarily palmitic and stearic acids). Due to the presence of oleic, palmitic, and stearic acids, cottonseed oil is often referred to as "naturally hydrogenated", making it a suitable frying oil that doesn't require additional processing or produce trans-fatty acids.

The cottonseed oil industry asserts that cottonseed oil requires less hydrogenation compared to other polyunsaturated oils to achieve similar results. Additionally, refined and deodorized cottonseed oil is considered to be one of the purest food products available. Despite being highly refined, it still maintains its nutritional quality (List & King, 2006).

Gossypol is a toxic, yellow, polyphenolic compound produced by cotton and other members of the order *Malvaceae*, such as okra, which facilitates natural insect resistance. In the cottonseed oil refining process, the refining, bleaching and deodorizing steps act to remove the gossypol level

Coconut oil is composed of a significant amount of glycerides with low chain fatty acids and exhibits high resistance to atmospheric oxidation. Its chemical properties are characterized by a low iodine value, high saponification value, and a high content of saturated fatty acids. Moreover, it remains in a liquid state at average room temperatures of 27°C.

Aside from fatty acid glycerides, natural fats also contain minor amounts of other substances. These unsaponifiable constituents are mainly sterols. Coconut oil, for example, contains small quantities of tocopherols and phytosterols as unsaponifiable components.

Effects of ingredients of vegetable origin use in trout feed

Since 1974, algae have been added to the list of healthy foods by the United Nations World Food Conference due to its nutritional properties.

Microalgae hold significant potential as a sustainable alternative to fishmeal and fish oil in aqua feeds. They can be cultivated using seawater or wastewater on arid, infertile land with minimal nutrient input, while achieving a net biomass production that surpasses any terrestrial plant or animal. Microalgae biomass can accumulate high levels of protein (40-70%) and lipids, which are essential for fish growth and development. Additionally, they contain numerous value-added components such as carbohydrates, vitamins, antioxidants, probiotics, carotenoids, and amino acids that enhance fish health and quality (Shahin et al., 2023).

Specifically, *A. platensis* is considered a superfood (Jung et al., 2019) due to its antiviral, antibacterial, antioxidant, anti-diabetic, anti-cancer, and anti-inflammatory properties. This microalga has been shown to enhance fish development (Roohani et al., 2019), stress tolerance, and resistance to hunger, making it an effective supplement in fish farming (Nandeesha et al., 1998; Kumar et al., 2022). As a result of its beneficial properties, *A. platensis* has been increasingly used in fish diets to improve fish welfare.

Algae feeding experiments in aquaculture have been conducted to evaluate their potential as protein sources and as additives in fish feed, as a complete replacement for fishmeal. The use of algae as an additive has been found to have a positive effect on fish, as it can lead to lower levels of cholesterol and fat, and improve lipid metabolism (Holman & Malau-Aduli, 2012).

According to an experiment conducted on 216 rainbow trout for 10 weeks, Iranian researchers have concluded that *A. platensis* (spirulina) can serve as an alternative natural source of carotenoids instead of synthetic astaxanthin in the diets of rainbow trout. The researchers found that the inclusion of 7.5% *S. platensis* was sufficient to ensure pigmentation without any negative impact on fish growth (Teimouri et al., 2013a).

One study aimed to assess the possible protective effects of *A. platensis* on rainbow trout specimens exposed to three different doses of the toxicant CdCl2. Cadmium is a highly toxic heavy metal that is widely distributed in the environment. Unlike essential heavy metals like iron and zinc, cadmium is a non-essential element that can accumulate in the body and cause harm even at very low concentrations. Due to its toxic nature, cadmium exposure is a major concern for human health and the environment. It can enter the food chain through contaminated soil, water, and air, and has been linked to various health problems including kidney damage, bone demineralization, and cancer (Cicik & Engin, 2005). The exposure of *O. mykiss* to CdCl2 resulted in alterations in serum and liver function biochemical parameters, reductions in antioxidant enzyme activities, and an increase in markers of oxidative stress. However, Banaee et al., in 2022, showed that dietary supplementation of *A. platensis* was effective in minimizing or eliminating the negative effects caused by the heavy metal. The inclusion of *A. platensis* in the fish diet normalized all altered serum and blood parameters induced by CdCl2 exposure and had a protective effect on oxidative stress markers. These findings suggest that supplementing the diet of farmed fish with *A. platensis* may enhance their stress tolerance, which could improve their wellbeing, quality, and yield in aquacultural production systems.

Roohani et al. (2019), developed a complete randomized experimental design to assess the effect of dietary spirulina inclusion in fish meal sparing (FMS) on slow-growing juvenile Caspian brown trout. Fishes fed spirulina diets, despite the fish's sensitivity to diet composition had meaningfully higher body mass gain and specific growth rates compared to those fed the control diet, as well as higher protein and lipid efficiency and lower feed conversion ratio. Whole-body composition analysis revealed higher protein and lower lipid content in fish fed spirulina diets, with the highest protein deposition and lowest lipid content reported in this group. Spirulina supplementation also resulted in higher levels of beneficial fatty acids in fish fillet and increased fillet and skin color parameters. The study concluded that spirulina treatment improved growth, carcass composition, and pigmentation in juvenile Caspian brown trout.

A. platensis has been increasingly used as a dietary supplement due to its protein content and positive contributions, such as reducing oxidative damage (Mahmoud et al., 2021). Sheikhzadeh et al. (2019) found that feeding *Oreochromis niloticus* with algae-based feed resulted in improved antioxidant biomarkers, particularly in the gills and liver. The aim of 2019 study, was to investigate the effects of *A. platensis* on various parameters in rainbow trout, including growth, fillet composition, and mucosal antioxidant activity in the intestine, skin, and gill. The administration of 2.5% spirulina significantly increased total antioxidant activity in all three mucosal tissues, while feeding total antioxidant activity in the bowels were higher when fishes received doubled spirulina quantity. Gene expression analysis showed that adding 2.5% and 5% spirulina in forage, can upgrade the physiology of intestinal and skin tissue, in terms of catalase, glutathione S-transferase gene expression, antioxidant parameters and glutathione peroxidase action.

In the diets of three species of salmonids (*Salvelinus fontinalis, O. mykiss, Salmo trutta fario*), the fishmeal-based feed was completely replaced with *A. platensis*. There were observed differences in spirulina acceptance and conversion among the species, with the experimental diets being well-accepted except for brown trout. A species-diet interaction was observed, resulting in a reduction in final body weight due to spirulina supplementation for brook and rainbow trout ($p < 0.05$). Furthermore, the feed conversion ratio increased to the same extent in the spirulina-fed fish ($p < 0.05$), indicating that both species had similar abilities to convert the spirulina diet (Rosenau et al., 2022). The study found that there are changes to the colour and fatty acid profile of the fish. The colour of the fillets became more yellow and red due to the pigments found in spirulina, and this change was observed in both raw and cooked fillets. Additionally, the fatty acid profile of the fish was altered, with an increase in saturated and monounsaturated fatty acids and a decrease in polyunsaturated fatty acids. However, the study also found that the complete replacement of fishmeal with spirulina led to a reduction in growth and a decrease in feed conversion efficiency. Overall, the study suggests that using spirulina as a complete replacement for fishmeal may have some negative impacts on production performance and product quality traits.

Spirulina is a viable replacement for fishmeal, but it changes the colour of the fillet to yellow. This should not be seen as a disadvantage, but rather an opportunity to increase the perceived quality of the product. Studies show that consumers prefer the yellow colour of fillets produced with spirulina, making it not only a sustainable source of protein for fish, but also a way to increase the value of trout fillets. Fillet colour is the second most important factor when searching for fish fillets, after freshness, but the country of origin is the most important factor affecting consumer preferences. It is unclear how consumers would perceive traditional red fillets produced by feeding astaxanthin compared to the yellow/orange fillets produced with spirulina (Habib et al., 2008).

Teimouri et al. (2013b) conducted an experiment to investigate the impact of algae powder on concentration of carotenoids in blood (BCC). The findings indicated increasing the levels of *S. platensis* in the diet resulted in a significant increase in BCC. Fish fed with over 7% *S. platensis* had a higher concentration of carotenoids in blood paralleled with other meals. Positive correlations appear between concentration of blood carotenoids and development and average daily growth. In terms of food conversion and BCC it was observed negative relationship. In the muscle regression analysis showed that blood carotenoid levels were positively related to their final levels. Also, when rainbow trout were fed with *S. platensis*, carotenoids concentration in blood was highly correlated with the colour of fillets. The study found no significant difference in fillet carotenoid content after two weeks of storage at 4°C. Additionally, the carotenoid content in fish fed *S. platensis* remained stable at 4°C and at least for three months at -20°C. However, after six months, a significant decrease was observed in frozen storage. All these results indicated that the value of carotenoids in the blood will influence the final colour of the trout fillets, and during the storage of these food products the carotenoids will have a more stable state if the fish are fed with *S. platensis* (Teimouri et al., 2013b). Later researches suggested that low-level spirulina supplementation can increase the amount of beneficial polyunsaturated fatty acids (PUFA) (Teimouri et al., 2015; Roohani et al., 2019). However, with higher exchange rates of fishmeal with spirulina, this effect was reversed, implying that there is a limit to the amount of spirulina supplementation that can be utilized to avoid undesired reduction of desirable PUFA (Jafari et al., 2014).

It is worth noting that the quantity of spirulina included in fish feed can vary depending on the specific product and the intended purpose. The optimal dosage of spirulina for trout is not well-established and may depend on factors such as the age and size of the fish, as well as the specific nutritional needs of the fish. Some studies suggest that algal biomasses may not be highly effective as substitutes for rainbow trout diets due to their limited use of vegetal nutrients. The current experiment confirms this trend, as rainbow trout fry fed with a feed containing more than 12.5% algal biomass showed lower growth performances (Dallaire et al., 2007).

Another species of unicellular microalgae that can be used as feed in fish food is *Nannochloropsis oceanica*. There are studies that try to find out what would be the advantages and disadvantages of its use in trout.

In 2020, Sarker et al. investigated the effects of incorporating a mixture of microalgae meal (*Schizochytrium limacinum* and *N. oceanica*) into the diet of saltwater-reared rainbow trout. Three experimental diets with varying levels of microalgae meal were tested over a 10-week period. The results showed that increasing the inclusion of microalgae meal had a negative impact on growth performance and feed conversion ratio. However, it also led to improved liver health. The study suggests that microalgae meal can be included in rainbow trout diets as a sustainable replacement for fish oil, but higher inclusion levels may negatively affect growth performance.

Although there are studies that show that digestibility is lower at Nanno, this disadvantage can be overcome through processing technologies, which overcome the complex structure of the cellulosic wall and the high fiber content (Sarker et al., 2020). Various processing techniques, including extrusion and enzymatic treatment, were assessed for the *N. oculata* co-product. Extrusion processing at a lower temperature (90°C) yielded superior outcomes in digestible protein and amino acids compared to high-temperature extrusion (127°C). *N. oculata* was utilized in its raw form, as well as after treatment with enzymes and exposure to extrusion processes. The obtained product showed a good potential for improving protein and energy digestibility and also for essential amino acids, and omega-3 polyunsaturated fatty acids (n-3 PUFA) (Sarker et al., 2023).

Rapeseed meal is a commonly used food with essential nutrients for many fish species, but its great fibber volume confines its nourishing importance for predatory fish (McCurdy & March, 1992; Burel et al., 2000; Shafaieipour et al., 2008; Kaiser et al., 2022). Still, as when rapeseed meal is included in fish diets at rates lower than 50%, the fibre content is unlikely to exceed 8% of the diet, which is unlikely to impair growth performance in fish (Hilton & Slinger, 1986). Fish species, particularly carp, have been observed to have better tolerance for glucosinolates compared to swine and poultry. In the case of trout, the recommended upper limit for glucosinolates in the diet is set at 1.4 µmol/g, indicating their sensitivity to higher levels. Consequently, including rapeseed meals with low glucosinolate content in the diet can be beneficial, with recommended inclusion rates ranging from 20% to 30%. This approach ensures adequate nutrition for fish while minimizing any potential negative effects of glucosinolates (Feed Industry Guide, 2019). The mixture of rapeseed and soybean is often used as an alternative to fish meal in fish diets because they are good sources of protein and can replace the essential amino acids found in fish meal. In addition, the use of plant protein reduces the cost of the diet and also eliminates the risk of dioxin and PCB contamination, which is a concern for many consumer (Hertrampf & Piedad-Pascual, 2000; Newkirk, 2009). Studies have shown that the digestible energy content of rapeseed meal is lower than that of soybean meal in salmonids, with values ranging from 9.6-11.5 MJ/kg as fed, while soybean feed has an estimated digestible energy content of around 13.0 MJ/kg in its as-fed form. This value indicates the amount of energy that can be efficiently utilized by animals during digestion and metabolism (Sauvant et al., 2004; National Research Council, 2011).

The digestibility of rapeseed protein is high, ranging from 83% to 99%, making it an excellent source of amino acids, particularly for Atlantic salmon. In comparison to other plant-based protein sources, rapeseed protein has the most favourable amino acid profile (Anderson et al., 1992). Although rapeseed has been widely studied in rainbow trout, it has usually been established to have a negative effect on performance (Hilton & Slinger 1986; Burel et al., 2000; De Francesco et al., 2004; Drew et al., 2005; Newkirk, 2009; Alami-Durante et al., 2010; Collins et al., 2013). A study conducted on juvenile rainbow trout showed that including rapeseed meal in their diet at levels of 10%, 20%, and 30% during a 9-week period, had negative repercussions on the hepatosomatic indices, growing rate, feed conversion percentage, and state of immunity of the fish (Hernández et al., 2013). While most studies have found that rapeseed meal has a negative impact on the growth and performance of rainbow trout, there are studies which shown different results. For instance, some studies found that rapeseed meal covering 25 μmol/g glucosinolates could be included in supplies for young rainbow trout at up to 30 percents, without affecting growth, feed intake or feed efficiency (Burel et al., 2001). Another study found that rapeseed meal could be incorporated at 17.5% of the diet's dry matter, in combination with soybean meal at 14.5%, to substitute 40% of the protein delivered by fish meal. However, overall, rapeseed meal is not a preferred protein source for rainbow trout diets due to its negative impact on growth and performance (Güroy et al., 2012).

Canola meal is commonly used in the diets of salmon and trout; however, its inclusion is restricted due to the high protein requirements of these fish and the presence of heat-stable anti-nutritional factors. To evaluate the impact of different inclusion rates of canola meal, a meta-analysis was conducted using data from 12 studies and 30 data points focusing on the effect of canola meal in rainbow trout diets. The findings revealed that including canola meal at rates of up to 20% did not have a significant effect on the growth rate of the fish. This suggests that moderate inclusion of canola meal in the diets of rainbow trout is well-tolerated and does not compromise their growth performance (Collins et al., 2013).

The demand for commercially reared fish and crustaceans has increased, leading to a deficiency of fish oil, which in the future is predicted to worsen. As a result, there has been a move towards substituting fish oil with vegetable oils, which has been well-documented and typically has minimal effects on fish growth performance (Turchini et al., 2013).

Commonly used vegetable oils in salmon and trout diets are canola oil and rapeseed oil, due to their unsaturated fatty acids levels, with omega-3 and omega-6 fatty acids. In particular, due to its low levels of the omega-6 fatty acid linoleic acid, which helps to maintain a natural omega-3:omega-6 ratio found in fish, canola oil, is highly wanted. Vegetable oils are used extensively as a replacement for fish oil in fish diets. It has been shown to have little impact on growth performance in fish. Fish oil can be replaced in diets for rainbow trout, with canola oil, up to 90%, without adverse consequence in growing and developing performances. Additionally, in fillets, the total omega 3: omega 6 proportion, showed only minimal changes. Canola oil is therefore a suitable replacement for fish oil in fish diet (Turchini et al., 2013; Masiha et al., 2015).

Studies have shown that replacing up to 100% of the supplemental lipid in rainbow trout diets with canola oil does not have a significant impact on fish performance. This indicates that canola oil can be used in aqua feeds, as a suitable alternative to fish oil, without affecting growth or feed efficiency (Karayücel & Dernekbaşi, 2010).

Rainbow trout fish fed with different oilseeds (*B. napus*) had the final weight and weight gain significantly lower comparing to those fed with the control diet ($P<0.05$). However, fish fed with *B. rapa* and *B. carinata* at a 15% inclusion level had similar final body weight and gain as the control. Overall, the study suggests that all three oilseeds have potential for use in rainbow trout feeds (Anderson et al., 2018). Rapeseed oil has no harmful impact on the growth of rainbow trout and Arctic char, and it does not significantly affect the total lipid content of their white muscle. However, in rainbow trout, different tissues composition in fatty acid is influenced by the regime, and the muscles were more affected than the liver. Triacylglycerols are more susceptible to changes than phospholipids. Lower levels of highly unsaturated fatty acids, such as eicosapentaenoic acid and docosahexaenoic acid were found in muscle and liver of rainbow trout fed with rapeseed oil have, resulting in an inferior n-3:n-6 proportion. On the other hand, in the muscle of rainbow trout, the content of E vitamin increases with higher levels of rapeseed oil in the food. Although rainbow trout prefer fish oil over vegetal oil, they do not show any preference among different levels of vegetable oil inclusion in their supply (Rosenau et al., 2023).

Carinata flour (*B. carinata*) replaced fishmeal with different amounts of at 50 g, 100 g, and 150 g levels in Kasiga & Brown study (2019).

The outcomes of the study exposed that there was no significant modification in body weight or visceral weight. A 2020 study investigated the use of carinata (*B. carinata*) and camelina (*C. sativa*) seeds to enhance the nutritional composition of Rainbow trout feed. However, the inclusion levels of raw carinata and carinata seed meals in animal diets are restricted by the presence of antinutrients, mainly glucosinolates (GLS), sinapine, and crude fiber (Kasiga et al., 2020).

The seeds were subjected to processes to improve the chemical composition by cold pressing, extrusion, solvent extraction and aerobic conversion and then administered in fish feed, where the palatability was evaluated, depending on the acceptability shown by the fish. Carinata meals were found to have a higher crude protein content compared to camelina meals and also, the fiber content was lower in the case of feed with carinata. Carinata is believed to enhance nutrient utilization and boost meal efficiency in fish, making it a favored choice among feed manufacturers over other options (Kasiga et al., 2020).

Masiha et al. (2013a) carried out a study to assess whether a source of dietary lipid can be changed with **flaxseed** oil (also known as linseed oil) for rainbow trout fingerlings (*O. mykiss*). The findings of the study suggested that the fingerlings could be successfully raised on diets where fish oil had been substituted with flaxseed oil, without any significant impact on fish performance.

Linseed oil is still under research due to the digestibility problems it may have. Yu et al., 2019, has concluded following a study from 2019 that the Manchurian trout may have the ability to synthesize LC-PUFAs from ALA, and an appropriate linseed oil in substitution of fishmeal oil (<75%) could improve both the lipid metabolism and the oxidation resistance.

In 2022, Dupont-Cyr et al. conducted a study to explore the potential of linseed oil as a complete substitute for fish oil in the diet of *Salvelinus alpinus*, *S. fontinalis*, and their reciprocal hybrids, all belonging to the Salmonidae family. The research aimed to assess the influence of dietary lipid source on muscle fatty acid composition, growth performance, and feed utilization across four experimental groups fed diets containing either 100% linseed oil or 100% fish oil. The study findings revealed that replacing fish oil with linseed oil did not significantly impact growth performance, feed utilization, or muscle lipid and protein content. However, the muscle fatty acid profile was notably influenced by the type of dietary lipid. Substituting fish oil with linseed oil resulted in a decrease in certain omega-3 fatty acids (20:5n-3 and 22:6n-3) and an increase in 18:3n-3. Even hybridization between the closely related species did not seem to affect the expression of key enzymes involved in highly unsaturated fatty acid (HUFA) biosynthesis. The study suggests that linseed oil can effectively replace fish oil in the diet of charr without adverse effects on growth, feed utilization, or muscle composition.

It is true that linseed oil does not improve the characteristics and composition of the meat, nor does it bring significant changes in fish growth. However, it can be considered when we want to replace fish oil with a sustainable ingredient.

Also, Masiha et al. (2013b) evaluated the appropriateness of **canola** (COD) and **flaxseed** oils (FxOD) as dietary lipid sources for rainbow trout fingerlings, significant differences in the fatty acid composition were observed among fish fed different lipid sources. The levels of linoleic acid and α-linolenic acid showed substantial increases in fish fed COD and FxOD, respectively. Interestingly, the study found that the concentration of α-linolenic acid in the muscle was lower compared to the diets, indicating a high degree of metabolism of this fatty acid through β-oxidation and/or desaturation and elongation processes in rainbow trout fingerlings. Despite a decrease in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) levels in the fillet of fish fed canola and flaxseed oils, the trout fillets remained a relatively rich source of these fatty acids, meeting the recommended daily intake of 500 mg/day of EPA plus DHA set by the International Society for the Study of Fatty Acids and Lipids. The study found that the fatty acid composition of rainbow trout flesh was influenced by the dietary fatty acid composition, and that fingerlings could be successfully raised on diets in which fish oil was replaced with canola and flaxseed oils. Additionally, the study concluded that the replacement of fish oil with these vegetable oils did not negatively impact the growth performance of the rainbow trout fingerlings (Masiha et al., 2013b).

Egg quality and reproductive performance of rainbow trout can be affected by various dietary oils. Fish oil (FO), linseed oil (LO), sesame oil (SO) and a commercial trout diet (CD) were fed to broodfish weighing approximately 870g, for about 5 months prior to reproducing period. Similar growing rate was among all fish studied groups. There were no significant differences between groups related to egg size. Moreover, the inclusion of LO or SO in the diet did not affect absolute fecundity, relative fecundity, or gonadosomatic index. As dietary lipid sources, LO and SO, can be used for broodfish and egg value and reproductive indices will have no negative changes. Additionally, efficient bioconversion of 18C fatty acids to 20-22C fatty acids was found in this study (Yıldız et al., 2020). The growth performance of rainbow trout was not impacted by diet, and there were only slight variations in semen volume, pH, and density ($p > 0.05$). Also, foods did not influence sperm microscopic qualities. Between the tested groups, semen osmotic values were different, higher values appear in groups fed with SO and LO. Sperm fatty acid summary, shows evidence of de novo biosynthesis of eicosapentaenoic acid, docosahexaenoic acid, and arachidonic acid. Despite this, reproduction indices were not significantly affected by food composition, suggesting that male rainbow trout are able to synthesize highly unsaturated fatty acids (Yıldız et al., 2021).

In 2011, Simmons et al., examined the impact on the quality of brook trout fillets when fishes are fed with **flax** diet. The study observed the impact of food adjustment using flaxseed oil-enhanced feed. The trout received either feed with fish oil (CD) or flaxseed oil (Flax) and then the fishes were harvested. The fillets were analysed for their proximate composition and fatty acid profile. During the storage period, raw fillets quality indices were also measured. Among the groups there were no significant changes, but the Flax fillets had a higher total omega-3 fatty acid content ($P<0.05$). Overall, the study suggests that a Flax-enhanced supply could have a positive impact on the brook trout bred in farms.

Czech researchers conducted an experiment on rainbow trout by adding 2% and 5% individually or a 5% mixture of sunflower or linseed oil to their diet. The firmness and juiciness of the fish meat were not affected by the addition of oils, but the taste of the fish was significantly influenced. The presence of sunflower or linseed oil did not have a significant effect on sensory characteristics, except for the intensity of the fishy taste. Linseed oil, which is a good source of n-3 polyunsaturated fatty acids (PUFA), contains a higher content of n-3 PUFA than sunflower oil, which can cause an off-flavour. However, none of the trout fillets were rejected due to the off-flavour. Both concentrations of linseed oil decreased the intensity of fishy taste. Therefore, the study recommends the use of linseed oil for the partial replacement of fish oil in rainbow trout feed (Drobná et al., 2006).

The effects of dietary supplementation of linseed (L) or sunflower (S) oil at 2.5% or 5%, or a mixture (5%) of both oils (LS5), was investigated on rainbow trout (*O. mykiss*). After 75 days of feeding, nutrient content in filleted fish was analysed. Weight gains, dry matter, fat, crude protein, cholesterol, saturated and monounsaturated fatty acids, arachidonic, eicosapentaenoic, and docosahexaenoic acids were not significantly different among the groups. However, meat from fish fed L5, S5, and LS5 had higher levels of polyunsaturated fatty acids (PUFA) compared to controls ($P<0.05$-0.01). The meat from fish fed L had less linoleic acid and more alpha-linolenic acid ($P<0.01$) compared to S-fed fish. S-fed fish had significantly higher levels of n-6 PUFA in their meat than all other groups. The concentration of n-3 PUFA was significantly ($P<0.05$-0.01) higher in the L-fed group compared to the S-fed group. The n-3/n-6 PUFA ratio in meat was significantly ($P<0.01$) higher in the L5 group compared to all other groups (Zelenka et al., 2003).

Adding 5% linseed or sunflower oils to extruded feed for rainbow trout, raised in recirculating systems, had several positive effects. It decreased the feed conversion ratio by 5.00% and 2.84% in trout fed EL and ES, respectively, compared to the control group. The weight gain of fish fed EL and ES increased by 5.03% and 2.14%, respectively, compared to fish in the C group. The inclusion of the oils did not affect the survival rate of fish in any of the groups, and it improved nutrient metabolism in rainbow trout. The study indicated that linseed and sunflower oils can successfully supplement extruded feed and replace some of the fish oil in rainbow trout diets. These oils did not negatively affect growth or survival rates but did improve weight gain and decrease feed conversion ratio. Vegetable oils in the diet increased the content of the essential fatty acids linoleic (LA) and α-linolenic (ALA), which can improve the lipid profile of the meat and be beneficial to human health (Zheliazkov, 2014).

In 2006, Italian researchers conducted a study on rainbow trout, exploring the possibility of herring and cod liver oil switching with coconut oil in the diet. The study lasted 231 days and involved four diets with varying levels of coconut oil, ranging from 0 to 13%. However, the study did not find any significant changes in the carcass features or meat composition of the rainbow trout fed with the different diets (Ballestrazzi et al., 2006).

Copra meal (derived from coconut) can be used as a feed ingredient for fish, but it is not an optimal one. This is because it contains less protein than fish meal or soybean meal and is lacking in lysine and sulphur amino acids. Although it is rich in arginine, excessive intake of dietary arginine can lead to lysine deficiency in animals, hence it is necessary to supplement fish diets with additional lysine and methionine to ensure adequate nutrition when using copra meal (Newkirk, 2009; Tacon et al., 2009). Additionally, it is important to note that phytic acid, tannins, and non-starch polysaccharides may appear in coconut oil as antinutritional combinations. These compounds can bind to nutrients, making them less available to the fish and reducing the overall nutritional quality of the diet. Therefore, careful consideration should be given to the inclusion of copra meal in fish diets and appropriate measures should be taken to minimize the negative effects of antinutritional factors (Tacon et al., 2009). Due to its high crude fiber content, copra meal is not an ideal feed ingredient for aquatic feeds. It is more suitable for herbivorous and omnivorous fish, where it can be included at rates ranging from 5-15%. However, for carnivorous fish, copra meal is less valuable and should be included at lower rates, typically between 5-10% (Hertrampf & Piedad-Pascual, 2000).

Despite the high melting point and saturated fat content of copra oil, which suggest it may be more suitable for warm-blooded mammals, it has been found to be an effective fat source in compound diets for the first-feeding larvae of common carp (*Cyprinus carpio* L.), even though they are reared at low temperatures (Fontagné et al., 1999; Fontagné et al., 2000a; Fontagné et al., 2000b). Studies have shown that including copra oil in the diet of rainbow trout did not have any negative effects on their growth (Figueiredo-Silva et al., 2012). Moreover, there were no adverse effects on reproductive performance in rainbow trout when copra oil was included in their diet (Ballestrazzi et al., 2006).

Luo et al. (2014) conducted a study on rainbow trout to investigate the effects of dietary fat source and level on plasma parameters related to health status. The experimental design involved feeding the fish high-fat (21%) or low-fat diets (11%) containing either highly saturated fat (derived from coconut oil - CO) or highly unsaturated fat (derived from fish oil - FO) as the sole fat source. The survival rate of the fish remained 100% during the three-week feeding trial for all dietary treatments. Final fish body weight and relative weight gain were significantly higher in the FO-High group ($P<0.05$) compared to fish fed the CO-High diet, with no significant difference observed between the FO-Low and CO-Low groups. Feed intake was also improved in the FO group compared to the CO group. Fodder productivity was however, not the same in all tests ($P>0.05$). According to a 15-week feeding study, the addition of CO in the rainbow trout meal did not result in a reduction in food intake. This finding is different from what has been observed in terrestrial animals, where the rapid oxidation and low retention of C12 in CO have been attributed to its satiating effect. In contrast, rainbow trout were found to deposit a significant portion of C12 and elongate/desaturate it into longer-chain fatty acids rather than quickly oxidizing it. This difference in fatty acid metabolism may explain why MCTs (medium-chain triglycerides) fail to create a satiety effect in rainbow trout

(Figueiredo-Silva et al., 2012). The ability of rainbow trout to efficiently include and convert C12, instead of rapidly oxidize it, differs from observations in mammals and may be the reason for the lack of a satiating effect of CO in this fish species.

In an experimental study, Lee et al., 2002, examined the efficacy of combining three diverse sources of processed **cottonseed** meal (CM) in the diets for juvenile trout. The objective was to completely replace fish meal (FM) protein with CM. The diets consisted of a combination of vegetal proteins (CM and soybean meal) and animal by-product proteins. The findings indicated that FM could be completely replaced with CM at a minimum level of 15% (equivalent to 25% replacement of fish meal protein) without any significant differences in growth rate and feed utilization. This suggests that incorporating CM as a replacement for FM in trout diets is a viable option without compromising the fish's growth performance and feed efficiency. However, in the control group haematocrit ranks were considerably higher compared with the group fed with CM-containing diets. The origin of the CM was found to affect its nutritive values in juvenile trout. Additionally, in the faeces of CM from Tennessee and Arkansas were found upper concentrations of total gossypol compared to that from California. The study also showed that gossypol optical isomer selectively collected in the liver and bile, while equal proportions optical antipode was established to be in the entire organism and faeces. Approximately 35-50% of dietary gossypol can be assimilated by fish, depending on the CM source, and the absorbed gossypol was almost completely eliminated (Lee et al., 2002).

Under the specific experimental conditions of a water temperature of 13 ± 1°C, researchers observed that juvenile rainbow trout with an initial average body weight of approximately 5.8 g had a mean feed conversion ratio (FCR) of 1.1 when fed diets containing either 100% fish oil or vegetable oils (such as soybean or sunflower oil). This indicates that the conversion of feed to body weight gain was relatively efficient, with similar FCR values observed for both fish oil and vegetable oil diets (Şener and Yıldız, 2003). Complete or partial replacement of fish oil with cottonseed oil in diets for rainbow trout did not have a negative impact on growth and feed utilization. The composition of fish fillet and total lipid levels in the liver of the fish is significantly affected. The feed conversion ratio (FCR) ranged from 1.3 to 1.4, indicate similar feed efficiency. However, in fish fed the cottonseed oil diet the hepatosomatic index (HSI) and viscerosomatic index (VSI) were higher paralleled to other supplies. Fish oil substitution with cottonseed oil led to a reduction in total fatty acid (n-3 PUFA) ranks and an increase in total n-6 PUFA levels in the fish fillet. Overall, the study demonstrated that replacing 50% of fish oil with cottonseed oil is feasible for rainbow trout diets, without compromising growth performance or fillet composition (Güler & Yildiz, 2011).

CONCLUSIONS

Fish meal and fish oil can be substituted in the diets of many fish species as long as all essential nutrients it provides are supplemented from other sources. This requires ensuring that the diet includes adequate levels of proteins, amino acids, lipids, vitamins, and minerals essential for fish growth, health, and development. Various plant-based ingredients, along with other sources like insect meal and yeast, can be used to create a balanced diet. These alternatives can be formulated to meet the nutritional needs of fish, thereby maintaining optimal growth rates, feed conversion ratios, and overall fish health.

The use of plant-based ingredients can help to reduce the environmental impact and cost of trout farming while maintaining the nutritional quality and growth of the fish.

Among different plant-based sources, algae, including spirulina, have shown promising results as a dietary supplement for trout, providing a source of protein, essential amino acids, vitamins, minerals, and antioxidants. Spirulina supplementation has been shown to improve growth rate, feed utilization, and immune function in trout.

Other plant-based sources that have been investigated for use in trout feed include Brassica sp. (such as canola), cotton, coconut, flax, and sunflower. These ingredients can provide a source of protein, energy, and essential fatty acids. Studies have shown that these ingredients can be included in trout feed at various levels without adversely affecting growth or survival. In the researched bibliography, studies were found regarding other species of oleaginous plants, which can be used in fish feeding, such as canola oil, palm oil. It should also be taken into account the possibility of mixing different types of vegetable feed to partial or total replace fish oil. Overall, the use of plant-based ingredients in trout feed shows promise as a sustainable and cost-effective alternative to traditional animal-based ingredients. However, it's important to carefully evaluate the quality and nutritional composition of plant-based ingredients and to optimize their use in trout feed to ensure optimal growth and health of the fish. Further research is needed to determine the most effective formulas and possibilities to mix different types of vegetable origin ingredients, for trout feed.

To ensure the sustainability of trout farming and aquaculture in general, it is essential to consider the environmental impact and ethical aspects of fish meal production and explore innovative and sustainable alternatives in fish feed formulation.

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SPATIO-TEMPORAL VARIATIONS OF LENGTH, TOTAL WEIGHT AND BODY CONDITION INDEX OF THE MEDITERRANEAN HORSE MACKEREL FROM THE ROMANIAN BLACK SEA AREA

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Abstract

The purpose of this study was the analysis of spatio-temporal variations of length, total mass and the Fulton condition factor (useful index for monitoring feeding intensity) as well as of the length-total mass relationship, which is an important parameter, which helps to know the growth pattern of fish populations. A number of 1200 individuals from the Trachurus mediterraneus species (Steindachner, 1868) that were collected from pelagic trawls and stationary uncovered pound nets along the Black Sea coastal area in the periods 2013-2015 and 2018-2020 were analysed. The average lengths and weights were 11.4 ± 2.3 cm and 14.0 ± 8.3 g, respectively, and the minimum and maximum values were 3.0 and 19.0 cm in length and 0.6 and 61.2 g in weight. The value of exponent b in the equation is 2.9485, indicating that the weight increase is allometrically positive. The mean value of the Fulton was 0.84 ± 0.11, and the minimum and maximum values were 0.42 and 1.74. The average length and average weight were significantly correlated with the Fulton index (r = - 0.069; p = 0.017, respectively r = 0.168; p < 0.0001). The total length and the Fulton index of horse mackerel varied significantly between stations and between years and months, which is most likely due to seasonal variations, as well as trophic differences between areas.

Key words*: biometric measurements, Fulton index, length-weight relationship, statistical analyses.*

INTRODUCTION

The ichthyofauna of the Black Sea has undergone significant changes in the last 60 years, both in the qualitative and quantitative structure, as well as in the behaviour of the different species (Nicolae et al., 2018). These changes are, most probably, the consequences of human activities, both directly through fishing pressure and indirectly through the deterioration of environmental conditions. The Black Sea basin is characterised by the fact that most fish occupy large areas in exclusive areas of the riparian countries and make feeding and spawning migrations throughout the basin. In this connection, the Romanian coastline occupies an important place, being known for its role in feeding and reproduction of the main species, although the biomass of catches obtained in this area is quite low.

The continuing decline in populations of many fish species, including horse mackerel, is mainly due to the deterioration of the habitats necessary for their survival. In a few decades, the intensification of many human activities, such as agriculture, industry, energy, transport and tourism, has led to the loss or fragmentation of natural habitats (Yatsu, 2019). For centuries, traditional forms of agriculture or traditional fishing activities have represented a way of efficient resource management (FAO, 2020). The abandonment of these activities and their application on an industrial scale, without studying the resilience limits of natural fish populations, has led to a depletion of stocks, biodiversity and changes in the landscape.

The horse mackerel *Trachurus mediterraneus* (Steindachner, 1868) is a member of the Carangidae family and represents a large part of the amount of fish caught in the Black Sea basin (Cocan & Mireșan, 2018). Species of the *Trachurus* genus are pelagic fishes of economic importance. A small pelagic species, the horse mackerel is also important to Romanian fisheries for economic and social reasons.

The biological characteristics of horse mackerel have been previously characterised in different areas of the Black Sea. Prodanov et al. (1997) studied the growth and estimated the optimum exploitation level of horse mackerel along the Bulgarian coast. Yankova & Raykov (2006) reported data for growth parameters and natural mortality coefficient of this species in Turkiye. Ambroaz (1954) investigated the distribution, migratory patterns, and catch composition. The biology of this species along the Romanian coast was reported in 1979 by Cautis & Ionescu and by Păun et al. (2019a; 2019b; 2020; 2021). Despite the significant number of studies on horse mackerel, there is a lack of recent data on the morphometry and reproduction of this species on the Romanian coast.

This scientific research analyses a time series (2013-2015 and 2017-2020) of data from pelagic trawls and stationary fishing nets found along the Black Sea coast, with the aim of analysing the spatio-temporal variations of the main biological parameters (length and total weight and the condition factor).

In particular, length-weight relationships have important applications in fisheries science and population dynamics, such as length-weight conversion for biomass estimation, building stock assessment models and estimating fish health status (Froese, 2006). In addition, they allow the monitoring of seasonal variations in fish growth and the comparison of morphological traits and life history between species or populations in different habitats and regions (Richter et al., 2000). Indices or condition factors are widely used to study the biology of fish because they provide relevant information about the psychological state of fish, based on the principle that individuals of a given length that are heavier are in better condition (Craig et al., 2005).

Environmental fluctuations are thought to strongly influence the abundance of pelagic species and can also lead to changes in life history and growth patterns; therefore, data collected from long-term studies are essential for determining average growth parameters (Bellido et al., 2000).

This scientific research contributes to improving information on the biology of horse mackerel in Romanian Black Sea waters in order to develop sustainable management measures for these species (Păun et al., 2021).

MATERIALS AND METHODS

The study area was located in the northwestern and southern part of the Black Sea (Figure 1).

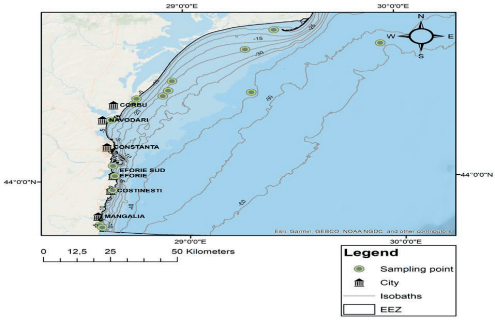

Figure 1. Study area (Original by ArcMap)

For this study, 1200 individuals were collected from pelagic trawls and uncovered stationary fishing nets along the Black Sea coast in the northern area (Gura Portiței, Periboina, Vadu, Corbu, Platforme, Cap Midia, Camp Năvodari Camp, Năvodari, Mamaia, Constanța) and the southern area (Agigea, Eforie Sud, Costinești, Olimp, 2 Mai, Vama Veche) in the 2013-2015 and 2018-2020 periods.

The fish caught were immediately refrigerated, packed in ice packs, and transported to the laboratory for measurements. Total length (TL), defined as the distance from the tip of the snout to the tip of the caudal lobes, was measured. Total length was measured to the nearest 0.1 cm and body weight (W) to the nearest 0.1 g. The instrument used to measure length was the ichthyometer, and an electronic scale with increased accuracy was used to determine weight. For some analyses, individuals were grouped into 1 cm length classes.

The length-weight relationship was used to determine the maintenance status of the fish and the type of growth (isometric or allometric). A coefficient b = 3 means linear

isometric growth of the fish (length and weight increase proportionally) (Ricker, 1975).
Statistical processing of the data was performed with the Statistica V12.7 software. First, stake data tables were created, bringing together individual and station codes, geographic coordinates (latitude, longitude), depth zone data, survey regions, sampling year and month, total weight (TW), total length (TL), and the annual variation of the Fulton index (Kn). Normality of data was tested using the Shapiro-Wilk test and homogeneity of variances was tested using the Levene test.
Depending on the results of these tests, parametric Anova or Ancova tests (covariance test) were used to analyse the effect of length separately from the effect of other factors: sex, seasons, years and months, Fisher (F) (if normality and homogeneity of variances were respected) or non-parametric Kruskal-Wallis (for more than two samples, H) or Mann-Whitney (for two samples, Z) tests (if normality or homogeneity of variances were not respected).
Significant tests (F, H or Z) and p-values (probability) have been indicated in the text and figure legends.
Comparison of pairs of values between sexes, stations, years and months was performed using multiple comparison tests of mean ranks or post hoc tests (Newman-Keuls or Bonferonni).
The Fulton index was calculated for the 1200 individuals sampled.
After taking biometric measurements and weighing individuals, the Fulton index (K) was calculated (Beverton & Holt, 1957), according to the following formula:
$K = (W/ l^3) \times 100$, where:
W = weight, in grams;
l = standard length, in cm.
Statistical analyses of individual Fulton index values were performed using STATISTICA 12.7 to analyse differences between sexes, stations, years and months.

RESULTS AND DISCUSSIONS

Distribution of sampled individuals

Trawl samples were collected only from the stations in the northern area i.e.: Gura Portiței, Periboina, Platforme, Corbu, Năvodari Camp, Mamaia and Constanța, and the stationary uncovered pound nets samples were collected from both the north stations: Vadu, Corbu, Camp Năvodari and from the south ones/ stations: Agigea, Eforie, Costinești, Olimp, 2 Mai and Vama Veche.
Of the 1200 individuals analysed, 671 were in the northern area and 529 in the southern area. The highest number of individuals sampled was in Corbu station (270) and the lowest number of individuals analysed was in Mamaia and Olimp stations (Figure 2).

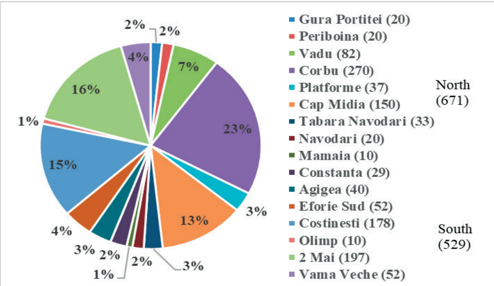

Figure 2. Distribution of sampled individuals per station

Most individuals were sampled in July 2018, the month with the highest number of individuals sampled over the entire study period, which may also be due to the breeding period of the horse mackerel (Figure 3).

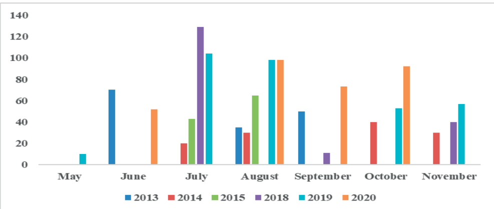

Figure 3. Distribution of individuals analysed by years and months

The lowest number of individuals sampled (10 individuals) was found in May in 2019 and also in September 2018 (11 individuals).
On the Romanian coast, the horse mackerel appears when the water temperature exceeds 13°C, usually in the last decade of May, and remains near the coast until autumn, in November, when the water temperature drops below 14°C.

Total length and total mass of horse mackerel

In this study a number of 1200 individuals were analysed. The average lengths and weights (± standard deviation) were 11.4 ± 2.3 cm and 14.0 ± 8.3 g, respectively, and the minimum and maximum values were 3.0 and 19.0 cm in length and 0.6 and 61.2 g in weight.

Their average lengths and weights were significantly correlated, the relationship between total weight and total length being: $W = 0.0094 \times TL^{2.9485}$, as it results in Figure 4.

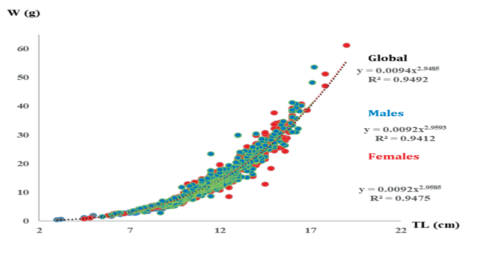

Figure 4. Relationship between total length (TL, cm) and total weight (W, g) in all horse mackerel analysed on the Romanian Black Sea coast in the period 2013-2020, males and females

In the case of horse mackerel individuals analysed for the period 2013-2015 and 2018-2020, the length-weight correlations had positive values. Correlation takes values between 0 and 1 R^2 (0;1) which can be converted to percentage values ($\sqrt{R^2}$).

The value of correlation becomes very important when values can be compared for a larger set of data and over a long period of time. Also, the closer the value of R is to 1, the tighter the correlation is (Fowler et al., 1998).

The length-weight relationship is used to determine the body condition of the fish and the type of growth (isometric or allometric). A coefficient b = 3 signifies linear isometric growth of the fish (length and weight increase proportionally) (Ricker, 1975). Thus, for males the equation is W = 0.0092-TL2.9593 and for females it is W = 0.0092-TL2.9585, thus showing a linear isometric growth pattern, undifferentiated by sex.

The length and total weight of males did not differ significantly from that of females ($p > 0.05$), but juveniles had a mean total length and mean total weight significantly lower than those of males and females (7.5 ± 1.3 cm and respectively 3.4 ± 1.2 g) ($H = 146.09$; $p < 0.001$ and respectively $H = 155.05$; $p < 0.001$).

Males and females were grouped as adults (TL = 11.6 ± 2.0 cm) for statistical analyses of spatial (between stations) and temporal variation across years and months.

Overall and for adults, depth of sampling stations from 2018-2020 was not correlated with total length and total weight ($p > 0.05$). For juveniles only, significant negative correlations appear, with larger juvenile individuals of greater size and weight collected at shallower depths ($r = -0.38$; $p = 0.004$ and $r = -0.52$; $p < 0.0001$, respectively).

Spatial variation of total length

Horse mackerel total length varied between stations ($H = 160.89$; $p<0.001$). The largest adults (males and females) were sampled at the Olimp station (14.4 ± 1.0 cm) and the smallest at the Năvodari station (9.5 ± 1.5 cm) ($H = 140.35$; $p<0.001$) as shown in Figure 5.

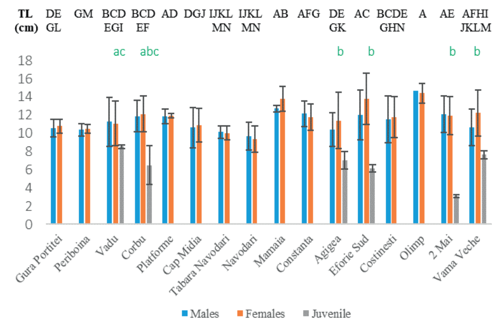

Figure 5. Spatial variation in total length (TL, cm) in adults (males and females) ($H = 140.35$; $p < 0.001$) and juveniles ($H = 45.07$; $p < 0.0001$)

Different letters indicate significant differences for adults (upper case) and juveniles (lowercase)

In juveniles, the largest were measured at the Vadu station (8.5 ± 0.2 cm) and the smallest at the 2 Mai station (3.1 ± 0.1 cm) ($H = 45.07$; $p < 0.0001$).

Annual variation in total length

The total length of horse mackerel showed inter-annual variations ($H = 318.81$; $p < 0.001$). The largest adults (males and females) were sampled in 2015 (13.8 ± 1.2 cm) and the smallest in 2014 (8.8 ± 1.5 cm) ($H = 331.26$; $p < 0.001$) as shown in Figure 6.

In juveniles, the largest were measured in 2020 (8.0 ± 0.7 cm) and the smallest in 2013 (3.1 ± 0.1 cm) ($H = 331.26$; $p < 0.001$).

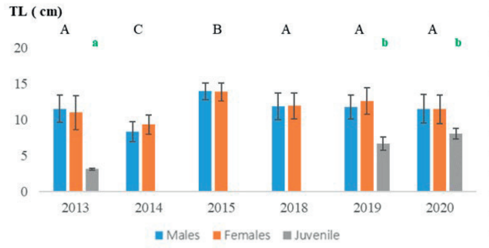

Figure 6. Annual change in total length (TL, cm) in adults (males and females), ($H = 331.26$; $p < 0.001$) and juveniles ($H = 26.82$; $p < 0.0001$)
Different letters indicate significant differences for adults (upper case) and juveniles (lower case)

Monthly variation of total length

The largest adults (males and females) were sampled in May (13.0 ± 1.0 cm) and the smallest in November (10.4 ± 1.5 cm) ($H = 111.51$; $p < 0.001$). In juveniles, the largest were measured in August (7.9 ± 1.2 cm) and the smallest in June (6.5 ± 2.1 cm) ($H = 20.36$; $p < 0, 0001$), as result in the Figure 7.

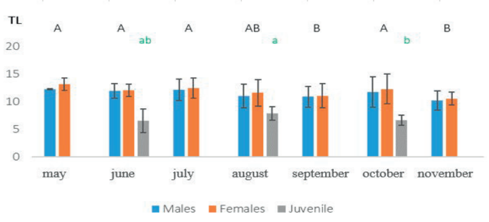

Figure 7. Monthly variation of the total length (TL, cm) in adults (males and females), ($H = 111.51$; $p <0.001$) and juveniles ($H = 20.36$; $p <0.0001$)
Different letters indicate significant differences for adults (uppercase) and for juveniles(lowercase)

Fulton index of relative body condition

The mean value of the Fulton index (± standard deviation) was 0.84 ± 0.11, and the minimum and maximum values were 0.42 and 1.74. Average length and average weight were significantly correlated with the Fulton index ($r = - 0.069$; $p = 0.017$, respectively $r = 0.168$; $p < 0.0001$).

The Fulton index in males was not significantly different from females ($p > 0.05$), but juveniles had a significantly lower index than males and females. (0.82 ± 0.22 cm) ($H = 22.68$; $p < 0.0001$). Males and females were regrouped as adults (0.84 ± 0.1 cm) for statistical analyses of spatial (between stations) and temporal variations of the Fulton index by year and month.

Globally, for both adults and juveniles, the depth of sampling stations from 2018-2020 did not correlate with the Fulton index ($p > 0.05$).

Spatial variation of the Fulton index

The Fulton's index of horse mackerel varied significantly between stations ($F = 9.50$; $p < 0.0001$), as shown in Figure 8, and as a function of individual length ($F = 19.04$; $p < 0.0001$).

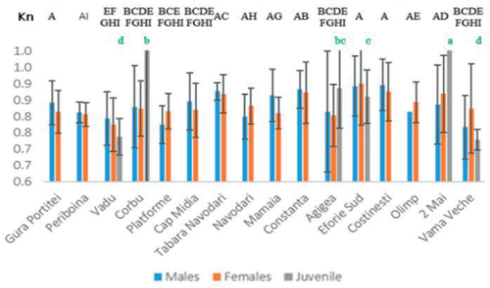

Figure 8. Spatial variation of the Fulton index (K) in adults (males and females),
($F = 7.82$; $p <0.0001$) and juveniles ($F = 15.39$; $p <0.0001$)
The different letters indicate significant differences for adults (uppercase) and juveniles (lowercase)

In adults (males and females), the highest values were measured at Eforie station (0.90 ± 0.11) and the lowest at Vadu station (0.78 ± 0.08) ($F = 7.82$; $p < 0.0001$). In juveniles, the highest Fulton index values were measured at 2 Mai station (1.74 ± 0.0) and the lowest at Vama Veche station (0.73 ± 0.03) ($F = 15.39$; $p < 0.0001$).

Their length also had a significant influence on the value of the Fulton index for both adults ($F = 7.66$; $p = 0.006$) and juveniles ($F = 41.44$; $p = 0.0001$).

Annual variation of the Fulton index

The Fulton index of horse mackerel showed inter-annual variations ($F = 33.73$; $p < 0.0001$), as well as depending on the length of individuals ($F = 17.12$; $p < 0.0001$).

In adults (male and female), the highest values were measured in 2018 (0.91 ± 0.09) and the

lowest in 2020 (0.79 ± 0.1) ($F = 32.32$; $p < 0.0001$).
In juveniles, the highest values of the Fulton index were measured in 2013 (1.74 ± 0.0) and the lowest in 2020 (0.75 ± 0.14) ($F = 14.84$; $p < 0.0001$) as shown in Figure 9.

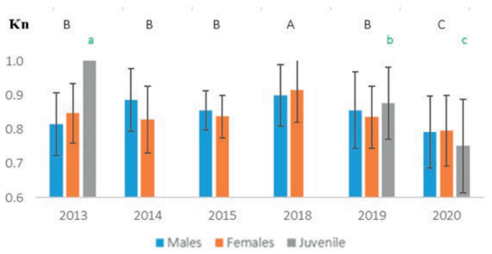

Figure 9. The annual variation of the Fulton index (Kn) in adults (males and females), ($F = 32.32$; $p <0.0001$) and juveniles ($F = 14.84$; $p <0.0001$)
Different letters indicate significant differences for adults (uppercase) and juveniles (lowercase)

Their length also had a significant influence on the value of the Fulton index both for adults ($F = 6.23$; $p = 0.01$) and for juveniles ($F = 28.58$; $p = 0.0001$).

Monthly variation of the Fulton index

The Fulton index of horse mackerel showed variations between months ($F = 23.02$; $p < 0.0001$) as shown in Figure 10, as well as depending on the length of individuals ($F = 19.67$; $p < 0.0001$).
In adults (males and females) the highest values were measured in May (0.94 ± 0.08) and the lowest in June (0.77 ± 0.08) ($F = 26.41$; $p < 0.0001$). In juveniles, the highest Fulton index values were measured in June (1.09 ± 0.64) and the lowest in August (0.78 ± 0.23) ($F = 4.27$; $p < 0.0001$).

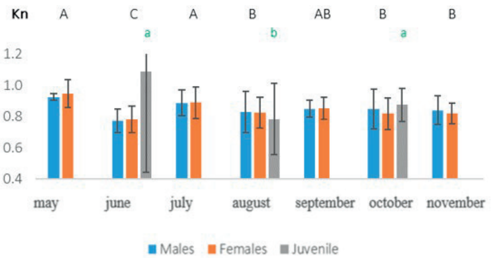

Figure 10. Monthly variation of the Fulton index (Kn) in adults (males and females), ($F = 26.41$; $p <0.0001$) and juveniles ($F = 4.27$; $p <0.02$)

Their length also had a significant influence on the value of the Fulton index for both adults ($F = 11.1$; $p = 0.001$) and juveniles ($F = 124.8$; $p = 0.0001$).
The average lengths and weights (± standard deviation) 11.4 ± 2.3 cm and 14.0 ± 8.3 g, respectively, and the minimum and maximum values 3.0 and 19.0 cm for length and 0.6 and 61.2 g for weight differs from those reported by Yankova & Raykov (2006) from the Bulgarian Black Sea coast (6.5-19 cm, respectively) and by Kasapoglu & Duzgunes (2012), respectively 13.53 ± 0.1 cm for the Black horse mackerel populations from the Turkish Black Sea coast (7.4-14.5 cm), as shown in Table 1.

Table 1. Comparison of average length and weight of horse mackerel with other studies in the Black Sea

References	Zone	Average length (cm)	Average weight (g)
Kasapoglu & Duzgunes, 2012	Black Sea (TR)	13.53 ± 0.1	20.72 ± 3.1
Ozdemir et al., 2015	Black Sea (TR)	13.02±0.02	18.24 ± 0.1
Kasapoglu, 2018	Black Sea (TR)	12..3 ± 2.8	-
Păun et al., 2021	Black Sea (RO)	11.5 ± 2.1	14.3 ± 8.3
Păun et al., 2019b	Black Sea (RO)	12.6 ±0.5	19.12 ± 0.5
The present study	Black Sea (RO)	11.4 ± 2.3	14.0 ± 8.3

However, there were differences in mean length values between investigations. According to Ozaydin et al. (2000), such differences can be attributed to the sampling strategy used, such as the sampling period, as well as temperature variations and likely differences in the trophic potential of the various areas.
Regarding the length-weight relationship, the values of coefficients a and b in other studies from the Black Sea were analysed and compared with those in the present study (Table 2).
The values of coefficient b, for horse mackerel, highlighted in the present study, do not differ significantly ($p > 0.05$) from the values identified in other studies.
Differences in b values can be attributed to one or more factors: season and the effects of different regions, differences in water

temperature and salinity, sex, food availability, differences in the number of specimens examined, as well as in the observed length ranges of the captured species (Goncalves et al., 1997, İşmen et al., 2009, Froese et al., 2011).
In 2019 and 2020, the number of juveniles increased significantly, this may be due to the overexploitation of the horse mackerel stock. A large proportion of immature and juvenile individuals below the optimum catch length (discarded catch) were taken by coastal fishermen from the stock and sold on the market under the counter or destroyed at sea. To eliminate this trend, which is an indicator of increasing overfishing, new fishing methods and management planning for horse mackerel stocks are considered necessary (Daskalov et al., 2012).

Table 2. Values of coefficients a and b in black horse mackerel (comparative analysis)

References	**Zone**	***a***	***b***
Șahin et al., 2009	Black Sea (TR)	0.0089	2.9552
Yankova et al, 2010	Black Sea (BG)	0.0035	3.3046
Yankova et al., 2011	Black Sea (BG)	0.0050	3.1680
Kasapoglu and Düzgüneș, 2012	Black Sea (TR)	0.0060	3.1040
Ozdemir et al., 2015	Black Sea (TR)	0.0057	3.1249
Kasapoglu, 2018	Black Sea (TR)	0.0050	3.138
Păun et al.,2021	Black Sea (RO)	0.0073	3.0546
Păun et al.,2019 b	Black Sea (RO)	0.0121	2.8633
Yankova et al., 2020	Black Sea (BG)	0.078	3.2412
The present study	Black Sea (RO)	0.0094	2.9485

Horse mackerel total length varied between months ($H = 108.79$; $p < 0.001$).
As Borcea (1928) also pointed out, larger specimens of the black horse mackerel, 13-20 cm in length, appear on the Romanian coast in spring; from July, the smaller specimens of 9-12 cm approach the coast, and from August, the youngsters of this species appear.
Similar to the Bulgarian Black Sea coast, Yankova et al. (2020), in their study found that the largest individuals sampled were in June and August (12.5 cm), and the smallest were found in November, their proportion dropping to 9-10 cm, which is due to juvenile individuals aged 0+.
Regarding the Fulton index, Yankova et al. (2020), in their study conducted on the Bulgarian Black Sea coast, found values of the Fulton index K = 0.804-0.828 in the analyzed period 2018-2019.
Yankova (2013), in her study, found that the value of the growth coefficient K increased (0.64 per year). This may probably be due to the average annual water temperature in the Black Sea during summer and partly to the dominance of age 2+ individuals in the catch. The sea surface temperature showed high cooperative values in the range 28-31 degrees Celsius (Roessig et al., 2004).
Sudden changes in temperature can have disastrous effects on fish populations (e.g. heat stress) (Abdulakarim et al., 2005). It is observed that the K value is higher in young (juvenile) fish.

CONCLUSIONS

The average lengths and weights in the present paper differ from those recorded on the Bulgarian and Turkish coast of the Black Sea. Such differences can be attributed to the sampling period as well as to temperature variations and likely differences in the trophic potential of the various areas.
Mean lengths and weights were significantly correlated, with the relationship between total weight and total length being W = 0.0094 x TL2.9485. The b-coefficient values, for horse mackerel, highlighted in the present study did not differ significantly ($p > 0.05$) from the values identified in other studies.
Horse mackerel total length varied between stations and between years and months. The largest adults were sampled at Olimp station and the smallest at Năvodari station. This may be due to the better trophic conditions in the southern area. The largest adults were sampled in 2015 and the smallest in 2014. Looking at the variation between months, we observed that the largest adults were sampled in May, when the horse mackerel approaches the Romanian Black Sea coast and the smallest in November, when it leaves the Romanian coast.

The mean value of the Fulton index in the present study does not differ from the one reported on the Bulgarian Black Sea coast. Average length and weight were significantly correlated with the Fulton index. The Fulton index of males did not differ significantly from that of females ($p > 0.05$). The Fulton index varied significantly between stations, months and years. For adults, the highest values were measured at Eforie station and the lowest at Vadu station, this could probably be due to the greater availability of food in the southern area than in the northern one. The highest values were observed in 2018 and the lowest in 2020. Regarding the monthly variation, we observe that the highest values of the Fulton index were in June, when the reproduction period starts and the lowest in August. Length also had a significant influence on the Fulton index value for both adults and juveniles.

Further research is recommended to improve the knowledge of horse mackerel population dynamics, seasonal migration between river basins (Black Sea, Danube) and influencing factors. There is a need for wider and more open communication between specialists, managers, decision-makers, fishermen, etc. to improve fisheries management.

ACKNOWLEDGEMENTS

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MORPHOLOGICAL PARTICULARITIES OF THE SNOW LEOPARD SKULL - IRBIS (*Panthera uncia* - Schreber, 1775) - CASE STUDY

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Abstract

The study describes the morphological characteristics of the panther leopard skull (Panthera uncia - Schreber, 1775). The skull particularities are valuable elements necessary for species identification. Following the analysis, the following conclusions emerged: the existence of an interfrontal fossa, elongated in an oro-aboral direction, located at the level of the interfrontal sagittal suture; the nasal extremity of the frontal ends with a sharp process; between the two processes of the rostral extremity of the nasals, lateral and medial, there is a wide and shallow incision; the naso-incisive incision is reduced; the mastoid process is reduced; a reduced accessory lacrimal foramen is present; between the maxillary and sphenopalatine foramen, an obvious vascular foramen can be observed; medio-aboral, on the edge of the jugular foramen is the hypoglossal canal; the pharyngeal tubercle is reduced and limited on the sides by two reduced fossae; the external occipital crest is well highlighted; above the ventral condyloid fossae are the deep and elongated dorsal condyloid fossae; the mental foramen is accompanied by an accessory mental foramen.

Ker words: *irbis, skull morphology, snow leopard.*

INTRODUCTION

The irbis or snow leopard (*Panthera uncia*) has its habitat in Afghanistan, the Lake Baikal region, eastern Tibet Plateau. It adapted over generations to live in the harsh, mountainous environment at altitudes of up to 5500 m. The snow leopard was included in the genus *Uncia* at the time of discovery. However, after numerous studies in which similarities with the genus *Panthera* were found, especially at the level of the hyoid bone, and after genetic research that highlighted its closeness to the tiger, it was included in the genus *Panthera* (Jackson, 2016; Johnson et al., 2006).

The IUCN in the *Red List* includes the species as vulnerable, with an estimated population of over 2,500 individuals and an estimated decline in the next three generations of at least 10%. In some areas where the species is protected, and its range remains intact, a stable or slightly increasing number of individuals is expected. Poaching is one of the major problems leading to species' decline.

According to research conducted in areas where poaching is very intense, it has been estimated that 221-450 snow leopards have been poached annually since 2008. If these estimates are relatively accurate, they suggest that approximately 2-10% of the snow leopard population may be poached annually (Nowell et al., 2016; Chundawat & Rawat, 1994; Alexander et al., 2015; McCarthy et al., 2017).

With all the data obtained from experts, it is complicated to estimate the size of the snow leopard population, as the lack of concrete data on general population trends prevents estimating the extent of any decline based on partial evidence, being largely speculative. However, the evidence does not show that the situation has deteriorated much since 2008, so this species has not been included in the *Red List* as an endangered species but only as a vulnerable species. Recent survey information indicates that snow leopard densities in several areas are higher than previously thought, implying that the total population size is also likely larger than minimum estimates (Jackson et al., 2010; Mallon et al., 2016; Berger et al., 2013).

Several conservation measures are being taken to reduce threats or mitigate their effects, including establishing new protected areas in the snow leopard range, more effective anti-poaching measures, training the conservation

professionals in Asian states with snow leopard populations; several independent initiatives to reduce conflicts with pastoralists; community engagement programs and educational programs to raise awareness of the snow leopard and its habitat.
Although the snow leopard is undoubtedly still threatened, the above measures have improved the overall conservation situation of snow leopards since 2008.
Studies have been carried out on the functional adaptability of the forelimbs for moving on rocky terrain in alpine areas, concluding that *Panthera uncia* is intermediate between *Acinonyx jubatus* (cheetah) and *Panthera onca* (jaguar). A scapular and pectoral musculature development provides stability to the shoulder girdle when the animal brings down large prey and supports it during jumping and climbing. At the *biceps brachii* muscle level, a unique bifurcation is observed in its tendon, which may provide increased functional stability at the level of the humeroradial joint (Smith et al., 2021). There are also differences at the level of brachial plexus between domestic cats and snow leopards, which may reflect different prey capture strategies between the subfamilies (Hall et al., 2023).
Numerous studies have been conducted on the conservation of the species in different habitats (Schaller et al., 1988), including the ovarian activity of female snow leopards throughout the estrous cycle and gestation through an enzyme immunoassay that measures fecal concentrations of estrogen and progesterone metabolites (Reichert-Stewart et al., 2014).
In 2022, Dinesh Kumar Jha et al. conducted a study to develop a species identification technique based on the cranial morphometry of Asian big cats. This technique could be used in forensic analysis of poaching cases (Jha et al., 2022).
Usually, the skull is the forensic sample through which the species can be identified; for this reason, the study aims to describe the morphological particularities of the *Panthera uncia* skull.

MATERIALS AND METHODS

A skull from an adult snow leopard (neutered male) (*Panthera uncia*), which died of natural causes, was used for morphology description.
The skull from the Anatomy discipline of the Faculty of Veterinary Medicine Bucharest collection was obtained after removing the soft tissues and then subjected to controlled maceration, washing, and degreasing.
The most interesting aspects were described and photographed. The description and identification are by Nomina Anatomica Veterinaria (N.A.V.) 2017.

RESULTS AND DISCUSSIONS

The skull of the snow leopard is characterized by a viscerocranium smaller than the neurocranium. The dorsal face of the skull is elongated, presenting a maximum height before the detachment of the zygomatic processes of the frontals.
The maximum width of the dorsal face is at the level of the zygomatic processes of the frontals. In the central portion, at the level of the interfrontal suture, an interfrontal depression (fossa) can be observed, elongated oro-aborally (Figure 1).

Figure 1. Dorsal side of the snow leopard (*Panthera uncia*) skull (original)
1. Nuchal crest; 2. Parietal; 3. External sagittal crest; 4. External occipital protuberance; 5. Zygomatic process of the frontal; 6. Interfrontal fossa; 7. Zygomatic; 8. Nasal; 9. Incisive; 10. Lateral process of the nasal; 11. Nasal notch

The zygomatic processes of the frontal are short, triangular in appearance and latero-ventrally directed. Rostral to the zygomatic process of the frontal, a small supraorbital notch is observed. At the rostral extremity, the frontal ends in a pointed nasal process.
The exocranial face of the parietals is convex oro-aborally and medio-laterally, being separated at the level of the median suture by a high and sharp external sagittal crest.
The external occipital protuberance is reduced in height, slightly thickened, and drawn aborally. The external sagittal crest departs from it in a rostral direction and divides at the suture site between the frontal and parietal in two weakly highlighted temporal lines. The temporal lines end at the level of the free extremity of the zygomatic process of the frontal (Figure 2).

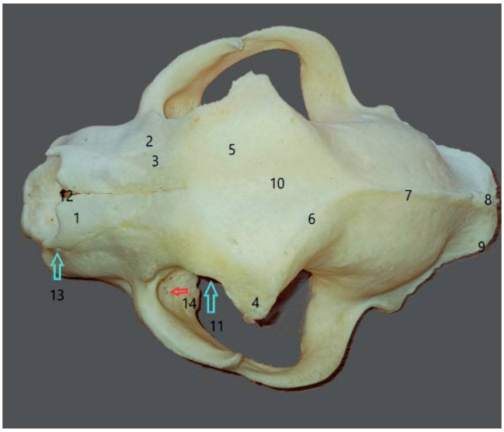

Figure 2. Dorsal side of the snow leopard (*Panthera uncia*) skull (original)
1. Nasal; 2. Frontal process of the maxilla; 3. Nasal process of the frontal; 4. Zygomatic process of the frontal; 5. Frontal; 6. Temporal line; 7. External sagittal crest; 8. External occipital protuberance; 9. Nuchal crest; 10. Interfrontal fossa; 11. Supraorbital notch; 12. Internasal notch; 13. Naso-incisive notch; 14. Maxillary foramen

The nasals are slightly concave in the aboral extremity and convex in the rostral one. Their rostral extremity is divided into two processes, one lateral elongated and pointed and a medial one short and rounded. A wide but reduced in-depth internasal notch is between the two nasal processes. The maximum width of the nasals is at their rostral extremity (Figure 3).
Between the nasal bone and the nasal process of the incisive is a reduced naso-incisive notch. The incisive body is evident, with a right angle shape; the upper part is horizontal, and the lower is vertical. The central (I1) and middle (I2) incisors are much smaller than the lateral incisors (I3). The entrance in the nasal cavity is wide, and the rostral portion of the vomer, which presents a deep septal sulcus, is easy to see (Figure 3).
The maxilla extends dorso-aborally through an obvious frontal process and shows a prominent canine alveolar relief on the lateral side.

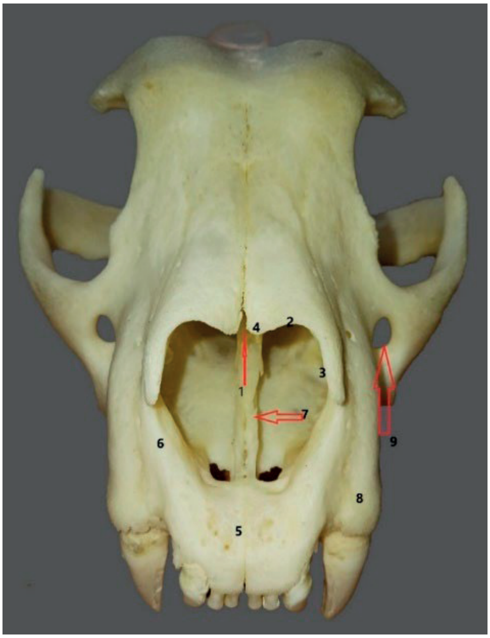

Figure 3. The rostral extremity of the snow leopard (*Panthera uncia*) skull (original)
1. Internasal notch; 2. Nasal; 3. Lateral nasal process; 4. Medial nasal process; 5. Incisive; 6. Nasal process of the incisive; 7. Vomer; 8. Canine alveolar relief; 9. Infraorbital foramen

The zygomatic process of the temporal is later-dorso-rostrally oriented and joins the zygomatic bone, forming the zygomatic arch (Figure. 4). The zygomatic arch is well-highlighted and has a lateral convexity.
The orbit is relatively circular, wide, and incomplete. The orbit communicates broadly with the pterygopalatine fossa.
The orbital hiatus is deep and presents the following openings: the ethmoidal foramen, the optic canal, the orbital fissure and the round foramen, missing the alar foramen and the alar

canal. The oval foramen can be observed ventro-aboral of the *foramen rotundum* (Figure 4 - 5).

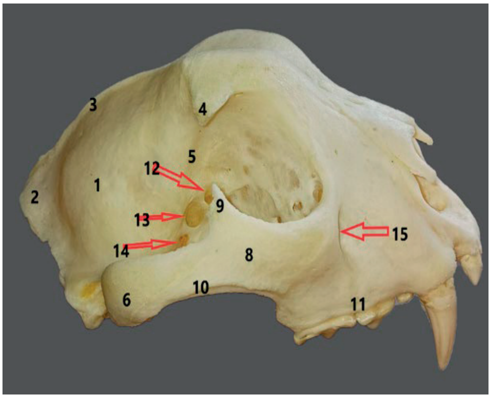

Figure 4. Lateral side of the snow leopard (*Panthera uncia*) skull – (original)
1. Parietal; 2. The external occipital protuberance; 3. External sagittal crest; 4. Zygomatic process of the frontal; 5. Orbito-temporal crest; 6. The zygomatic process of the temporal; 8. Zygomatic; 9. The temporal process of the zygomatic – the medial portion; 10. The temporal process of the zygomatic–lateral portion; 11. Alveolar process of the maxilla; 12. Optical canal; 13. Orbital fissure; 14. *Foramen rutundum*; 15. Infraorbital foramen.

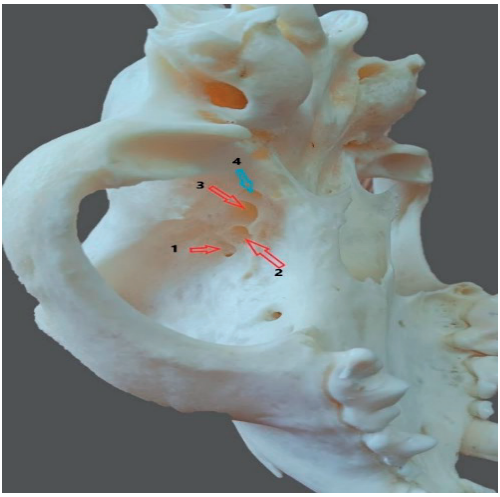

Figure 5. Lateral face of the snow leopard (*Panthera uncia*) skull – (original)
1. Ethmoidal foramen; 2. Optical canal; 3. Orbital fissure; 4. *Foramen rotundum*

A wide and shallow tympanic notch can be observed in the auricular region, and ventral to it is a wide and oval external acoustic meatus arranged slightly oblique caudo-rostral (Figure 6). The tympanic bulla is evident and oval, with convex surfaces arranged slightly oblique latero-medially.

Latero-aboral to the tympanic bulla is a reduced mastoid process, and at its base is a small stylomastoid foramen. Attached to the aboral extremity of the tympanic bulla is a short paracondylar process that is separated from the mastoid process by a small incision.

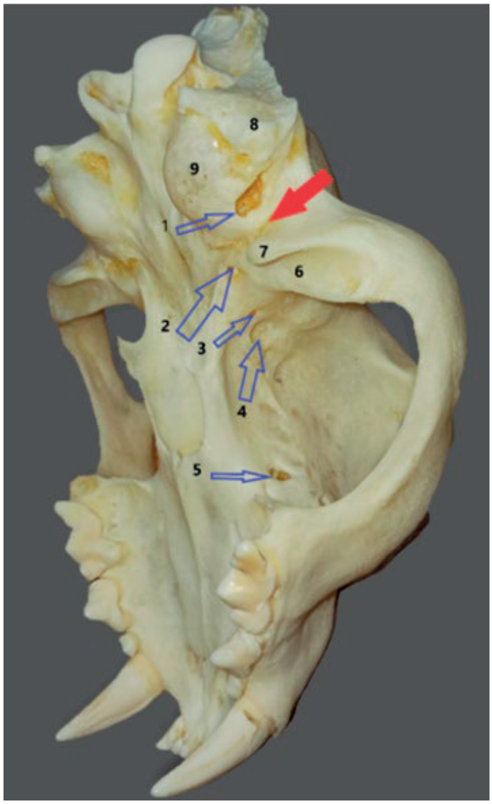

Figure 6. Lateral face of the snow leopard (*Panthera uncia*) skull (original)
1. External acoustic meatus; 2. Oval foramen 3. *Foramen rotundum*; 4. Orbital fissure; 5. Caudal palatine foramen; 6. Articular surface; 7. Retroarticular process-Retroarticular foramen (red); 8. Mastoid process; 9. Tympanic bulla

The maxillary hiatus has the following openings: the maxillary foramen, the sphenopalatine foramen, and the caudal palatine foramen (Figure 7). Between the maxillary and sphenopalatine foramina, there is an apparent vascular foramen.

The lacrimal fossa is reduced; there is an obvious lacrimal foramen at its level, and dorsally, it is a very small accessory lacrimal foramen.

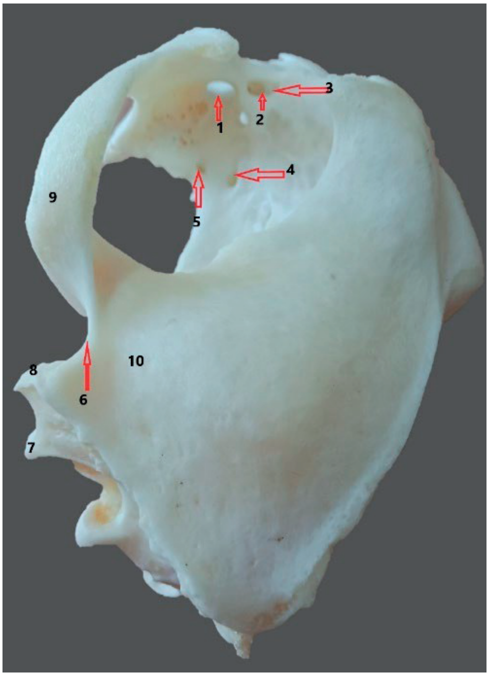

Figure 7. The laterodorsal face of the snow leopard (*Panthera uncia*) skull (original)
1. Maxillary foramen; 2. Lacrimal foramen; 3. Accessory lacrimal foramen; 4. Sphenopalatine foramen; 5. Caudal palatine foramen; 6. Tympanic notch; 7. Jugular process; 8. Mastoid process; 9. The zygomatic process of the temporal; 10. Temporal - squamos part

On the ventral face of the temporals' zygomatic process, the mandibular fossa is oro-aborally convex and elongated, and caudal to it is an obvious retroarticular process. A small retroarticular foramen is at the base of this process (Figure 6).
A defined jugular foramen can be observed laterally to the aboral extremity of the tympanic bulla, and the hypoglossal canal can be found medio-aborally on its edge (Figure 8).
On the exocranial face of the basioccipital is a reduced pharyngeal tubercle, bounded on each side by two reduced fossae (Figure 10).
An elongated and reduced fossa is in the central portion of the basisphenoid.
The pterygoid has a long, sharp, aborally oriented hamulus at the aboral extremity.
The spinous foramen can be observed on the rostral edge of the tympanic bulla and in the rostral direction, at a distance of less than 1 cm, the oval foramen. Lateral to the spinous foramen is the carotid canal (Figure 9).
The palatine processes of the maxilla are crossed at the base by the greater palatine foramina, which is continued orally with the well-marked palatine grooves up to their middle third.

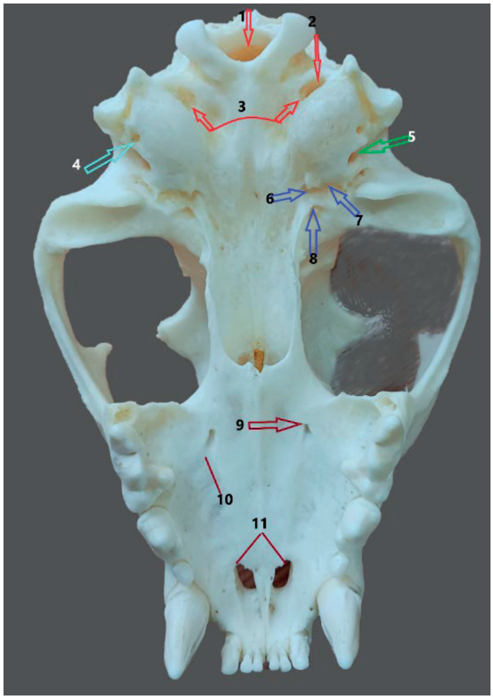

Figure 8. The ventral side of the snow leopard (*Panthera uncia*) skull (original)
1. Foramen magnum; 2. Jugular foramen; 3. Hypoglossal canal; 4. Stylomastoid foramen; 5. External acoustic meatus; 6. Spinous foramen; 7. Carotid foramen; 8. Oval foramen; 9. The greater palatine foramen; 10. Palatine groove; 11. Palatine fissures.

In the rostral extremity of the hard palate, there are two oval palatine fissures, which continue rostrally on the palatine surface of the incisive with two grooves that narrow in the rostral extremity of the incisive bone (Figure 8).
On each side of the foramen magnum is a slightly oblique dorsoventral condyle with the articular surface convex in the same direction. The spheno-basioccipital tubercles are inconspicuous. The jugular processes (paracondylar) are reduced and attached at the aboral extremity of the tympanic bulla (Figure 10).

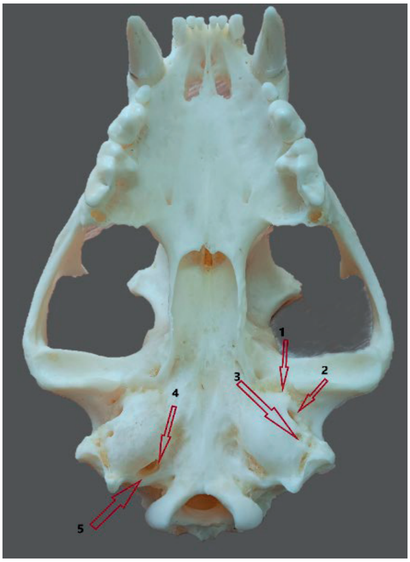

Figure 9. The ventral side of the snow leopard (*Panthera uncia*) skull– (original)
1. Carotid foramen; 2. External acoustic meatus; 3. Stylomastoid foramen; 4. Hypoglossal canal; 5. Jugular foramen.

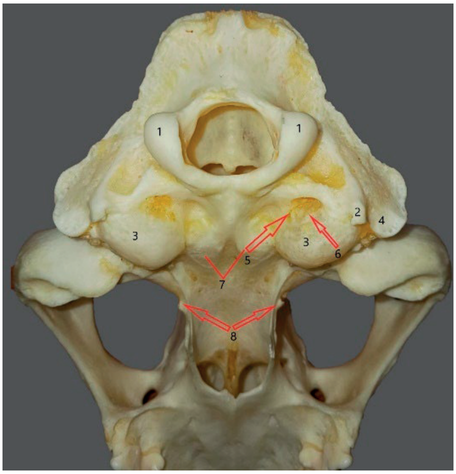

Figure 10. The nuchal face of the snow leopard (*Panthera uncia*) skull – (original)
1. Occipital condyles; 2. Jugular process; 3. Tympanic bulla; 4. Mastoid process; 5. Hypoglossal canal; 6. Jugular foramen; 7. Spheno-basioccipital tubercles; 8. Pterygoid processes

The nuchal ridges are well highlighted on the nuchal face, and the external occipital protuberance is reduced in height, slightly thickened, and drawn in the aboral direction.
From the external occipital protuberance descends a prominent external occipital crest. Rough lines of muscle insertions are present on either side of it (Figure 11).
The jugular processes are reduced and oriented ventro-aborally. The ventral condylar fossae are shallow, and the foramen magnum is wide and relatively rectangular.
Above the ventral condylar fossae are the deep and elongated dorsal condylar fossae. The two fossae, dorsal and ventral, are separated by a slightly oblique ridge.

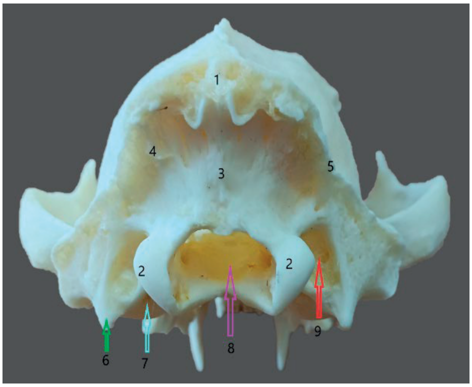

Figure 11. The nuchal face of the snow leopard (*Panthera uncia*) skull – (original)
1. External occipital protuberance; 2. Occipital condyles; 3. External occipital crest; 4. Lines of muscle insertion; 5. Nuchal crest; 6. Jugular process; 7. Ventral condylar fossa; 8. Foramen magnum; 9. Dorsal condylar fossa.

The mandible is an unpaired bone. The ventral margin of the horizontal portion is straight. The angular process is developed and slightly aboro-ventrally directed.

Figure 12. Snow leopard mandible (*Panthera uncia*) – lateral view (original)
1. Masseteric fossa; 2. Coronoid process; 3. Condylar process; 4. Angular process;
5. Mental foramen; 6. Accessory mental foramen

The masseteric fossa is deep and wide, exceeding half the height of the coronoid process. An accessory mental foramen accompanies the mental foramen (Figure 12).

The condylar process has a relatively cylindroid appearance with a convex articular surface. The coronoid process is curved aborally, and the free margin is relatively rounded.

Figure 13. Snow leopard mandible (*Panthera uncia*) – medial surface (original)
1. Coronoid process; 2. Condylian process; 3. Pterygoid fossa; 4. Angular process; 5. Mandibular notch; 6. Mandibular foramen; 7. Notch between the condylar and angular processes

On the mandibular medial surface is an obliterated pterygoid fossa, and dorsal to the angle is the mandibular foramen. Between the condylar and angular processes, a wide and shallow notch is observed (Figure 13).

CONCLUSIONS

At the level of the interfrontal suture, there is an interfrontal depression (fossa), elongated oro-aborally and rostral; the frontal ends with a sharp nasal process.

In the rostral extremity, the nasal bone is divided into two processes (one lateral and the other medial); between the processes, there is a wide internasal notch, reduced in depth, and between the lateral process of the nasal and the nasal process of the incisive there is a reduced naso-incisive notch.

The mastoid process, located latero-aboral to the tympanic bulla, is reduced, and at its base is a reduced stylomastoid foramen.

In the reduced lacrimal sac fossa, there is an obvious lacrimal foramen and, dorsally, a very small accessory lacrimal foramen.

In the maxillary hiatus open: the maxillary foramen, the sphenopalatine foramen, and the caudal palatine foramen; between the maxillary and sphenopalatine foramen, an obvious vascular foramen is present.

Lateral to the aboral extremity of the tympanic bulla, there is an apparent jugular foramen and medio-aboral, on its edge, the hypoglossal canal.

A reduced pharyngeal tubercle is present on the basioccipital's exocranial face, with two reduced fossae on either side.

On the nuchal face, from the level of the external occipital protuberance descends the external occipital crest, well highlighted and lateral to it are rough lines for muscle insertion.

The ventral condylar fossae are shallow, and above them are the deep and elongated dorsal condylar fossae, the ventral fossae being separated from the dorsal ones by a slightly oblique crest.

The angular process of the mandible is developed and slightly directed antero-ventrally. An accessory mental foramen accompanies the mental foramen. A wide and shallow notch is observed between the condylar and angular processes.

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INFLUENCE OF PROBIOTIC DIETS ON THE GROWTH AND WELL-BEING OF *Acipenser baerii* SPECIES IN A RECIRCULATING AQUACULTURE SYSTEM

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Abstract

In this study, the impact of a commercial probiotic (PC) on the growth and well-being of siberian sturgeon (Acipenser baerii, Brandt, 1869) in a recirculating aquaculture system was investigated over 63 days. Four experimental groups were created: a control group (A1) received a commercial feed with 45% protein and 15% lipid, while three others (A2, A3, A4) had the commercial feed supplemented with 0.2 g PC kg^{-1}, 0.4 g PC kg^{-1}, and 0.6 g PC kg^{-1}, respectively. Biometric measurements and blood analyses were conducted to assess growth and well-being of the biological material. Probiotic addition improved the growth performance in all experimental groups compared to the control. Erythrocytes, hemoglobin, and leukocytes showed higher mean values in the experimental groups. Serum glucose significantly decreased in the experimental groups (p<0.05), while total serum proteins increased with probiotic diets. Protein and lipid accumulation in muscle tissue was observed in all groups fed probiotic diets, indicating a positive influence on the growth and well-being of Acipenser baerii in recirculating aquaculture systems.

Key words: *aquaculture, growth, sturgeon, well-being.*

INTRODUCTION

Sturgeons are valued in aquaculture due to their high-quality meat and caviar production (Fontagné et al., 2006; Zarantoniello et al., 2021).
The Siberian sturgeon (*Acipenser baerii*) is a species with good adaptability to different growing conditions, has a shorter reproduction cycle compared to other sturgeons and is quite resistant to the stress factors that appear in the culture environment. These characteristics make it a good candidate for aquaculture (Bronzi et al., 2011; Dettlaff et al., 2012; Kolman et al., 2018).
Various sturgeon rearing systems are used in aquaculture. In 2016 estimates showed that 21% of all rearing systems were recirculating aquaculture systems (EUMOFA, 2020).
The disadvantage of these rearing systems, however, is that they can favor the occurrence and spread of certain fish diseases. The fact that fish are concentrated in relatively small spaces increases the risk of disease transmission between individuals high (Cristea et al., 2002). Recirculating aquaculture systems face challenges such as oxidative stress, digestive disorders and increased risk of bacterial or fungal infections.
The introduction of probiotics into the feed of fish reared in recirculating aquaculture systems is an important research aspect in the field of sustainable fish rearing in captivity and a promising strategy for improving growth performance and fish health. This is considering the benefits that these beneficial microorganisms can bring to the fish's digestive system (Verschuere et al., 2000; Ringø et al., 2006).
Probiotics, through their ability to colonize and modulate intestinal flora (Nayak, 2010), can help reduce these problems. They can secrete antimicrobial substances and stimulate the fish's immune system, making them more resistant to diseases (Li & Gu, 2018).
In addition to the health benefits of fish, introducing probiotics into their diet can also help improve feed efficiency (Hoseinifar et al., 2018; Neepa et al., 2022) and reduce negative

environmental impacts. By optimizing the digestion and absorption of nutrients, the amount of nutrients removed into the water from the recirculation system can be reduced, thereby reducing the risk of pollution and reducing the costs associated with water treatment.

Also, the application of probiotics in aquaculture can provide an alternative to the utilisation of antibiotics in disease treatment and prevention, thus contributing to the reduction of bacterial resistance and promoting a more sustainable and ecological practice in fish farming (Mondal et al., 2022).

In this context, this work aims to investigate the effects of supplementing the diet of the Siberian sturgeon (*Acipenser baerii*), reared in a recirculating aquaculture system, with a mix of probiotics (Probiotic Complex - PC), focusing on fish growth, welfare, and quality.

MATERIALS AND METHODS

The experiment was conducted at the Institute of Research-Development for Aquatic Ecology, Fisheries, and Aquaculture Galati, over a period of 63 days, aiming to evaluate the effects of a commercial probiotic (PC) composed of a mixture of bacteria (*Bifidobacterium lactis, Lactobacillus acidophilus, Lactobacillus salivarius, Lactobacillus plantarum, Lactobacillus casei*) as a supplement in the diet of Siberian sturgeon (*Acipenser baerii*). Accordingly, 120 specimens of Siberian sturgeon, with an average weight of 5.18±0.44 g, obtained through artificial reproduction at the Galati Institute, were randomly distributed into 4 growth units (A1-A4) of a recirculating aquarium system, as described by Savin et al. (2023). Water quality parameters were maintained at optimal levels using Tetra EX 1200 Plus external filters, supplemented with aeration stones. The water volume used was 125 liters, and it was refreshed daily at a rate of 50%. Temperature and dissolved oxygen were monitored daily using the portable analyser HQ40d by Hach Lange, while nitrogen compounds were measured weekly using the portable spectrophotometer DR1900 by Hach Lange and the LCK kits from Hach Lange.

Fish were manually fed twice a day with a basic feed, Aller Bronze type, with a granulation of 2 mm (Dynavit Impex, Brasov), containing 45% protein and 15% lipids. Three experimental variants and one control were tested, in which different amounts of probiotic were added to the basic feed; A1 (0 g PC), A2 (0.2 g PC / 5 x 10^9 CFU), A3 (0.4 g PC / 10 x 10^9 CFU), and A4 (0.6 g PC / 15 x 10^9 CFU). The powdered probiotic was dissolved in distilled water, mixed with a 2% gelatine solution, sprayed onto the feed, and dried at room temperature.

For assessing the efficiency of the probiotics used on growth performance, biometric measurements of the fish were conducted at the beginning and end of the experiment, calculating parameters according to established formulas:

Weight gain (WG) = final weight – initial weight (g)

Daily growth rate (DGR) = (final weight – initial weight) / day (g/day)

Specific growth rate (SGR) =100 x [ln (final weight) – ln (initial weight)] / days (%/day)

Feed conversion ratio (FCR) = feed intake / weight gain (g/g)

Protein efficiency ratio (PER) = weight gain / feed intake x crude protein of the feed

Blood samples were collected from the caudal vein at the end of the experiment. Prior to sampling, the fish were anesthetized by immersion in a solution composed of 2.5 ml of clove oil per 10 liters of water. For haematological analyses, blood was collected in heparinized Eppendorf tubes, while non-heparinized tubes were used for obtaining serum, which were left at room temperature for 30 minutes and then centrifuged at 4000 rpm for 5 minutes.

Red and white blood cells (RBC and WBC) were counted using the Neubauer counting chamber and the Potain pipette, employing Vulpian and Turk solutions as dilution liquids, respectively. The Kern OBN135 microscope, equipped with the Kern ODC 832 camera, was utilized for this purpose (Baker & Silverton, 2014).

The haematocrit (Ht) was determined by introducing blood into heparinized microcapillaries sealed with wax at one end, which were then centrifuged for 2 minutes at 10,000 rpm. Following centrifugation the haematocrit percentage was read directly on the haematocrit counter (Davison et al., 2023).

Haemoglobin (Hb) was determined using the cyanmethemoglobin method, in which the Drabkin reagent lyses the blood, forming a solution of cyanmethemoglobin. The absorbance was read on the UV-VIS spectrophotometer SPEKOL 1300 Analytikjena, at a wavelength of 540 nm (Kondi, 1981).
Serum glucose was measured using the Ortho toluidine method, with absorbance read on a spectrophotometer at 660 nm, while total serum proteins were determined using the biuret method (Kondi, 1981).
The total protein content was quantified using the Kjeldahl method, employing a Gerhardt-type system. Moisture content was measured by drying the sample in an oven at 130°C until a constant mass was achieved. Lipid content was determined using the Soxhlet method, which involves extracting fats with petroleum ether as a solvent, using a VELP-type extraction system. Ash content was estimated by calcining the sample in an oven at 600°C, followed by weighing the resulting residue.

The statistical analysis of the data was conducted using Excel 2019 and SPSS Statistics 17.0 software. To compare more than two groups, a single-factor ANOVA was employed, while a paired t-test was used for comparing two groups. Results were considered statistically different at $P < 0.05$.

RESULTS AND DISCUSSIONS

The addition of probiotics to the diet of the Siberian sturgeon did not significantly influence the water quality parameters (Table 1). Thus, the average dissolved oxygen values were within normal limits for fish growth (6.93-7.32 mg/l), with no significant differences between treatments ($p = 0.14$). The average temperature ranged between 21.05°C (A4) and 21.18°C (A1). Also, the values of nitrogen compounds showed non-significant differences between the experimental treatments, ranging from 0.09-0.1 mg/l (nitrites), and 4.46-4.77 mg/l (nitrates).

Table 1. Water quality parameters during the experimental period (mean ± SD)

Parameters	A1	A2	A3	A4
T (°C)	21.18 ± 2.25	21.13 ± 2.22	21.11 ± 2.13	21.05 ± 2.16
DO (mg/l)	7.32 ± 0.94	6.93 ± 1.01	6.93 ± 1.19	6.99 ± 1.11
NO_2 (mg/l)	0.1 ± 0.08	0.09 ± 0.08	0.1 ± 0.08	0.09 ± 0.08
NO_3 (mg/l)	4.77 ± 1.41	4.46 ± 1.15	4.47 ± 1.16	4.50 ± 1.20

The introduction of probiotics, in different concentrations, generally resulted in improved growth performance of Siberian sturgeon (Table 2). Enhancing the diet with 0.2 g PC per kilogram of feed led to a significant increase in growth rate in treatment A2 ($p<0.05$) compared to the control, while growth in treatments A3 and A4 was non-significant ($p>0.05$).

Table 2. Growth performance and feed utilization in Siberian sturgeon (*Acipenser baerii*) fed with different concentrations of PC

Parameters	A1	A2	A3	A4
Initial weight (g)	5.24	5.19	5.08	5.21
Final weight (g)	35.47	39.48	36.07	35.50
WG (g)	30.23	34.29	30.99	30.29
DGR (g/day)	0.48	0.54	0.49	0.48
SGR (%/day)	2.98	3.22	3.11	2.99
FCR (g/g)	0.64	0.59	0.62	0.65
PER (g)	0.70	0.76	0.72	0.69

At the end of the experiment, fish in groups A2, A3 and A4 had better daily growth rate and specific growth rate compared to the control. Also, the feed conversion factor (FCR) was improved in groups A2 and A3. However, in group A4 the FCR value was higher than that of the control group, suggesting that a higher concentration of probiotic could negatively affect the feed efficiency. Diet supplemented with probiotics had a positive impact on protein efficiency ratio (PER). There was a direct proportional relationship between the amount of probiotic added and FCR and an inverse proportional relationship with PER (Figure 1).

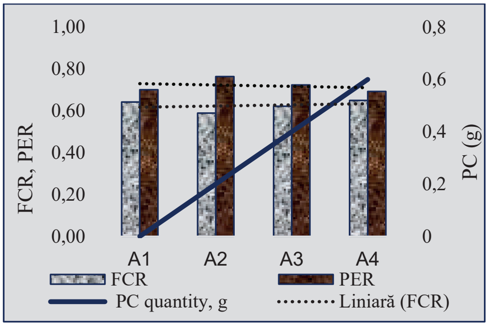

Figure 1. The evolution of FCR and PER during the experimental period

It is demonstrated that a healthy diet is very important for the healthy growth of an animal (Owens et al., 1993). Improved growth efficiency is associated with better nutrient digestibility (Al-Shawi et al., 2020).
In this study, the addition of probiotics to the feed at various quantities/concentrations positively influenced the efficiency of feed consumption. These results are consistent with those of other authors, who have demonstrated the beneficial effects of probiotics on growth performance (Perez-Sanchez et al., 2014; Tarkhani et al., 2020).
Proteins are among the most expensive nutrients required for the growth of organisms, and probiotics supplemented in the diet can help maximize their utilization. Probiotics can improve protein utilization efficiency by increasing the biological value of feed (Ringø & Gatesoupe, 1998).
According to Lara Flores and her colleagues (2003), supplementing diets with probiotics could have a significant effect in improving protein efficiency ratio (PER) (Lara-Flores et al., 2003).
In 2020, Hassani et al. supplemented the diet of Siberian sturgeon (*A. baerii*) juveniles with a mix of bacteria consisting of *Lactobacillus* spp., *Bacillus subtilis*, *and Bifidobacterium bifidum.* After 8 weeks of treatment, they observed the same effect of improving growth and feed efficiency (Hassani et al., 2020).

Improved growth performance has also been observed in other animals (Cakir et al., 2008; Kraimi et al., 2019).
The analysis of the haematological indices of the fish after the addition of feed with probiotics is important in establishing their health status (Fazio, 2019).
The effect of dietary probiotics on the blood indices of Siberian sturgeon (*Acipenser baerii*) juveniles is presented in the Figure 2. The use of the probiotic mix, named Probiotic Complex (PC), significantly improved the haematological parameters of the Siberian sturgeon ($p<0.05$). The experimental group A4, in which 0.6 g PC/kg feed (15×10^9 CFU) was added, showed the highest values of Hb, Ht, RBC and WBC, showing increases of 32.4%, 8.29%, 21.43%, and 13.85% respectively for these indices, compared to the control.

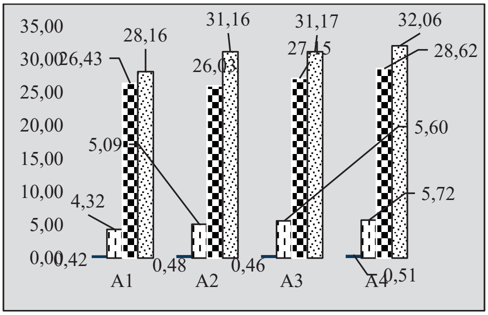

Figure 2. Variations in haematological parameters at the end of the experimental period

These results are consistent with those obtained in a previous study, where the erythrocyte count and haemoglobin level of Persian sturgeon (*Acipenser persicus*) were improved after treatment with *Pediococcus pentosaceus* and *Lactococcus lactis* (Soltani et al., 2016).
Also, Pourgholam et al. (2017) identified higher erythrocyte and haemoglobin values when supplementing the diet of Siberian sturgeon with *Lactobacillus plantarum.*
The biochemical parameters were also significantly improved upon supplementing the diet with PC ($p<0.05$) (Figure 3). The value of total proteins increased with the increase in the amount of PC added to the feed, being higher by 5.9%, 3.9% and 11.8% in A2, A3 and A4, respectively, compared to the control group. Regarding serum glucose, fish fed probiotic-supplemented diets showed lower blood glucose

levels compared to the control, indicating an optimal and healthy physiological state. The most difference compared to the control group was observed in A4, where the glucose level was 1.7% lower than in the control.

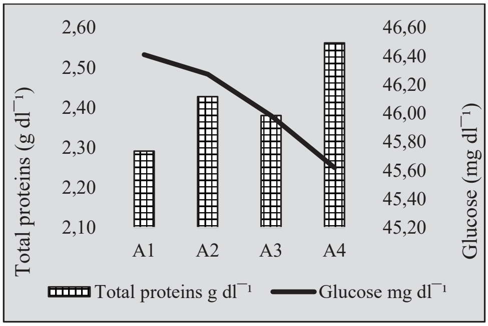

Figure 3. Variation in serum proteins and glucose of the *Acipenser baerii*

Similar results regarding the mean value of total proteins were obtained by Eissa et al. (2022) for sea bass fed diets supplemented with different amounts of *Pediococcus acidilactici*. However, contrary to the results of the present study, in their experiment, the mean glucose values were lower in the groups treated with probiotics than in the control group (Eissa et al., 2022).

Serum glucose also decreased in rainbow trout (*Oncorhynchus mykiss*) when feed was treated with *Bacillus subtilis* (Kamgar et al., 2014).

Regarding the biochemical composition of the meat, significant differences were observed between the control and the experimental groups (table 3). The crude protein content was significantly higher in probiotic treated groups (A2-A4) ($p<0.05$), while the moisture content decreased with the increasing probiotic content. Also, the analysis of the composition of the fish meat indicated higher percentages of lipids in the experimental groups compared to the control, the values increasing with the amount of probiotic included in the feed.

These results confirm those of Eissa et al. (2022).

Table 3. The biochemical composition of the meat of the Siberian sturgeon (*Acipenser baerii*) at the end of the experimental period

Parameter / Experimental group	A1	A2	A3	A4
Crude protein % (mean ± sd)	14.19±0.06	14.50±0.01	14.61±0.02	14.51±0.01
Lipids % (mean ± sd)	7.99±0.07	9.13±0.02	9.56±0.01	11.28±0.03
Moisture % (mean ± sd)	76.29±0.12	75.27±0.06	74.09±0.04	72.99±0.03
Ash % (mean ± sd)	1.20±0.01	1.03±0.02	1.16±0.04	1.16±0.05

Yones et al. (2019) obtained similar results when testing different levels of sorghum supplemented with Lacto cel-con probiotic in Nile tilapia (*Oreochromis niloticus*).

The increase in the protein content of fish meat could be explained by the fact that probiotics, once in the intestine, help to improve digestion and more efficient feed consumption, stimulating the body's growth. This leads to a greater accumulation of feed protein in the meat (Mehrabi et al., 2012; Noshair et al. 2023).

Although the results obtained in this experiment, together with previous studies have demonstrated the beneficial potential of probiotics in aquaculture, it is important to continue research to better understand how they can influence fish and their rearing environment. The results of this study could contribute to the development of sustainable and efficient practices in the farming of Siberian sturgeon and other fish species in similar aquaculture systems.

CONCLUSIONS

In conclusion, it can be stated that the inclusion of the bacterial mix from Probiotic Complex has improved the growth, health and quality of Siberian sturgeon meat.

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THE INFLUENCE OF SELECTED ENVIRONMENTAL FACTORS ON COMMON CARP (*Cyprinus carpio*) EMBRYONIC DEVELOPMENT AND HATCHING

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Abstract

Changes in the electromagnetic environment and increasing pollution of the environment are significant threats to animals including fish. In this study we made an attempt of determining some effects of geomagnetic field disturbances (hypogeomagnetic conditions), 1800 MHz electromagnetic field, and a commonly used herbicide (Roundup - the concentrations applied in the experiment corresponded to 0.1; 0.5 or 5 mg/l of the active ingredient) on common carp (Cyprinus carpio) embryonic development and hatching. In the periods of 24 and 48 hours after the start of incubation and then in every 6 hours till the end of the experiment the percentage of dead eggs, the percentage of hatched larvae, and the percentage of deformed larvae were determined. The research conducted showed that the deprivation of the geomagnetic field accelerated the hatching of larvae; a reduction in hatchability was also observed, while exposure to the electromagnetic field resulted in an increase in the percentage of finally hatched larvae (statistically insignificant changes). Roundup exposure resulted in increased mortality, reduced hatchability, and an increased percentage of deformed larvae (statistically significant changes).

***Key words**: fish, embryogenesis, EMF, GMF, herbicide.*

INTRODUCTION

The Earth electromagnetic environment including the geomagnetic field (GMF) is a permanent element of the environment, which was present during the phylogenetic development of all living organisms (Rochalska, 2009; Hulot et al., 2019). Then it seems possible that disturbances in this environment could have an impact on the course of life processes of organisms living on Earth. The industrialization of the environment causes increasing threats from artificial magnetic and electromagnetic fields generated by various devices (Fey et al., 2019; Formicki et al., 2021). Disturbances of natural electromagnetic environment may affect some physiological processes related to directional reactions taking place in embryos and larvae of fish (Tański et al., 2005; Formicki et al., 2019). The study conducted by Formicki and Winnicki (1998) showed that additional magnetic field (MF) significantly lengthened fish embryonic development. Moreover, artificial magnetic fields can affect water management processes in fish eggs (Sadowski et al., 2007).

In modern societies the environmental electromagnetic fields (EMF) are increasingly supplemented by anthropogenic EMF. Sakuli et al. (2000) studied the hatching process in zebrafish (*Danio rerio*) embryos treated with MF (50 Hz AC, 1000 µT). They showed that the EMF exposure did not affect the number of deformed larvae and the small hatching delays observed were not detrimental to the embryos.

On the other hand metal elements which are parts of buildings (construction and interior) could weaken the Earth natural magnetic field, which may not be neutral for living organisms (Tsunomura and Tokumoto, 2005). Tombarkiewicz et al. (2018) showed that weakened geomagnetic field influences embryogenesis and dynamics of hatching in Prussian carp (*Carassius gibelio*). The results obtained in the study conducted by Kantserova et al. (2017), who investigated the effect of hypogeomagnetic conditions on Ca^{2+}-dependent proteinases of fish, indicate that these enzymes are capable of direct perceiving of MF, however the effect of GMF on biochemical processes in living organisms has not been sufficiently studied so far.

Growth of civilization causes not only changes to the electromagnetic environment, but also chemical contamination. One of the most serious ecological problems is pesticide pollution of water environment. As shown in an extensive review by Bojarski and Witeska (2020), pesticides can get into groundwater and may affect fish. Alterations in haematological indices or blood biochemical parameters indicate that pesticides may cause pathophysiological changes in these animals (Bojarski & Witeska, 2020). Exposure to herbicides may also result in histopathological changes including hyperplasia and hypertrophy of gill epithelium and changes in liver microstructure, such as vacuolization of hepatocytes (Bojarski et al., 2018). Herbicides are the most commonly used pesticides (Cooper and Dobson, 2007; De et al., 2014). Despite the common use of such agrochemicals, the existing data regarding the effects of herbicide pollution of water ecosystems on fish early life stages is not sufficient.

The goal of the present research was to determine the effects of 1) hypogeomagnetic conditions 2) electromagnetic field (1800 MHz) exposure 3) exposure to herbicide Roundup on common carp (*Cyprinus carpio* Linnaeus, 1758) developing eggs. Embryo mortality, hatching dynamics, and percentage of deformed larvae were analysed.

MATERIALS AND METHODS

Artificial spawning

Artificial spawning was performed using common carp (*C. carpio*) R3R8 laboratory line, which were kept in recirculating aquaculture system (RAS). The fish were stimulated using Ovopel (Interfish, Hungary). Each pellet (50-55 mg) contains 25 μg of GnRH oligopeptide analogue and 20 mg of metoclopramide. In the first injection males received 1/2 a pellet per kg body weight, while females received 1/5 a pellet per kg body weight. In the second injection which took place 12 hours after the first injection only females were injected. They received 1 pellet per kg of body weight. After detecting ovulation symptoms, the animals were subjected to artificial spawning.

Incubation

In order to carry out the experiment oocytes taken from 10 females and sperm taken from 3 males were used. Oocytes from all females were mixed. Similarly, sperm from all males were mixed. Next, fertilization was conducted. Fertilized eggs were placed on glass Petri dishes (96 to 362 eggs per every dish in the GMF deprivation part of the experiment, 65 to 144 eggs per every dish in the EMF part of the experiment, and 78 to 141 eggs per every dish in the Roundup part of the experiment). Dechlorinated and aerated tap water was used in the experiment. The water in the dishes was changed every 12 hours. Physicochemical parameters (pH 7.6-7.8, NO_3^- 2-5 mg/L, NO_2^- 0 mg/L, NH_3 0 mg/L, GH 16-18 dGH, KH 8 dKH, PO_4^{3-} 0 mg) were controlled with aquaristical kits produced by Zoolek company (Poland). In the periods of 24 and 48 hours after the start of incubation and then in every 6 hours till the end of the experiment the percentage of dead eggs, the percentage of hatched larvae, and the percentage of deformed larvae were determined.

Geomagnetic field deprivation (hypogeomagnetic conditions)

The control dishes were maintained in an ambient GMF (about 37 μT), while the experimental dishes were kept in special cages with GMF weakened below 12 μT. Hypogeomagnetic conditions were achieved by using cages made of steel (the S235JRG2 type; CMC, Zawiercie, Poland). The construction of the cages was previously described by Roman and Tombarkiewicz (2009). To provide similar lighting conditions, Petri dishes of the control group were placed in cages which does not disturb GMF. Each group (control and experimental) contained 15 dishes.

Electromagnetic field exposure

The control group (15 dishes) contained eggs kept in standard conditions, while experimental eggs (15 dishes) were exposed daily to electromagnetic field. The exposure time was 26 min per day (13 times per day between 6:00 and

23:00 at equal time intervals, 2-minute emissions). The EMF field source was a generator of radio frequency electromagnetic waves. The output power was delivered to a Yagi GSM ceiling antenna with omnidirectional characteristics. The petri dishes were placed in an area where the average electric field intensity was 5.73 V/m (±0.84 V/m), the average magnetic field intensity was 0.016 A/m (±0.010 A/m), the average power density was 0.014 W/m^2 (±0.009 W/m^2) and frequency was 1800 MHz.

Roundup exposure

The eggs kept in the control Petri dishes were not exposed to herbicides or other chemicals. The experimental ones were treated with Roundup 360 Plus (Monsanto), a widely applied pesticide, which contains glyphosate (in the form of potassium salt) as an active substance. The concentrations used in the current study corresponded to 0.1, 0.5 or 5.0 mg/l of the active ingredient (respectively: group R1, R2 and R3). Each group included 20 dishes.

Statistical analysis

The Shapiro-Wilk test was applied for testing the compliance of the analysed data with the normal distribution. The Levene's test was applied for testing the homogeneity of variances. The level of significance was set at $\alpha = 0.05$.

Embryo mortality and hatching dynamics were analysed using two-way mixed ANOVA. The significance level of each test was set at 0.05. If the two-way mixed ANOVA did not show statistical significance for the group factor, no post-hoc analysis followed. If the statistical significance was found for the group factor, a series of one-way ANOVA for each time point was performed. At a given time point, a statistically significant difference was recognized provided that p.adj < 0.05, where p.adj was the product of p.value from the given one-way ANOVA test and the number of comparisons in the series. The number of comparisons was 7 for hatching dynamics in the experiment with Roundup and 22 for embryo mortality in the same experiment. In the other cases (hatching dynamics and embryo mortality in experiments on GMF deprivation and exposure to EMF), performing a post-hoc test was not necessary. Only the time points corresponding to the statistically significant differences found by one-way ANOVA tests described above (p.adj <0.05) were taken into account. Post-hoc analysis was continued using a series of t-tests for independent groups at selected time points. At every such time point only the control group was compared to the groups R1, R2 and R3, respectively. The Bonferroni correction for three comparisons was applied for the interpretation of each test result, that is, the test was statistically significant for p.adj <0.05, where p.adj = 3 * p.value and p.value is the result of a given t-test. The percentage of deformed larvae in each of the three experiments performed was analysed using a one-way ANOVA test. The significance level of each test was set at 0.05. In the case of statistical significance, a series of t-tests for independent groups was performed as a post-hoc analysis, where only the control group was compared with the groups R1, R2 and R3, respectively. The Bonferroni correction for three comparisons was applied for the interpretation of each test result, that is, the test was statistically significant for p.adj < 0.05, where p.adj = 3 * p.value and p.value is the result of a given t-test.

RESULTS AND DISCUSSIONS

Geomagnetic field deprivation (hypogeomagnetic conditions)

Embryo mortality at the time points in the control group and the group exposed to the hypogeomagnetic conditions were similar (Figure 1) - statistical analysis showed that the group factor was not significant (p = 0.0820).

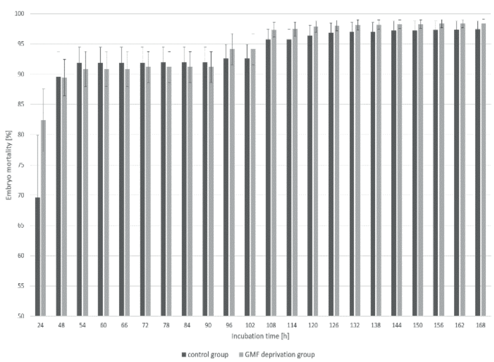

Figure 1. The effect of hypogeomagnetic conditions on embryo mortality rate of common carp (*Cyprinus carpio*)

On the other hand, the studies by Tombarkiewicz et al. (2018) showed that incubation of Prussian carp (*C. gibelio*) eggs under hypogeomagnetic conditions resulted in increased mortality (the difference between the experimental and control groups was statistically significant after 24 and 72 hours).

Even though an acceleration of the hatching process was observed in the GMF deprivation group (the beginning of hatching after 96 hours of incubation in the experimental group and after 108 hours in the control group) (Figure 2), the statistical analysis did not show a significant difference between both groups in terms of hatching dynamics (p = 0.871).

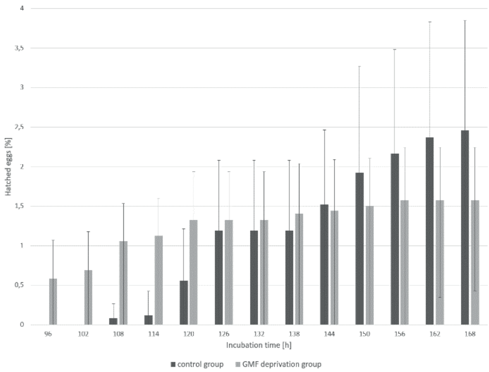

Figure 2. The effect of hypogeomagnetic conditions on hatching dynamics of common carp (*Cyprinus carpio*)

It is to be noted, however, that the statistical analysis did not include the observations after 96 and 102 hours of incubation due to the fact that in the case of the control group all values were zero. Generally, 56.24% more larvae hatched in the control group than in the experimental group. The results obtained in the experiment are partially in line with the results obtained earlier by Tombarkiewicz et al. (2018) who observed that hatching of Prussian carp (*C. gibelio*) larvae started 6 hours earlier in hypogeomagnetic conditions; however, the percentage of hatched larvae in the experimental group at the end of the experiment was higher than in the control group by 13%.

The percentage of deformed larvae in both groups (Fig. 3) were similar (p=0.951). Moreover, Tombarkiewicz et al. (2018) revealed that the ratio of deformed to non-deformed Prussian carp (*C. gibelio*) larvae of the control group and the GMF deprivation group were comparable. On the other hand, Asashima et al. (1991) observed that a shielded environment (5 nT) caused a significantly higher percentage of Japanese fire belly newt (*Cynops pyrrhogaster*) deformed embryos.

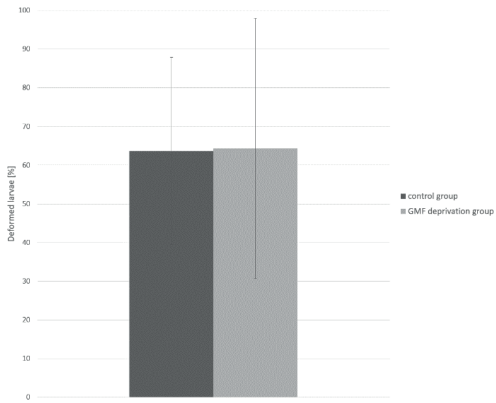

Figure 3. The effect of hypogeomagnetic conditions on the occurrence of deformations in common carp (*Cyprinus carpio*)

Electromagnetic field exposure

Embryo mortality at individual time points in the control group and the group exposed to EMF was similar (Figure 4) - statistical analysis showed that the group factor was not significant (p = 0.0910). Moreover, Li et al. (2014) showed that exposure to extremely low frequency magnetic field (ELF-MF) during incubation of zebrafish (*D. rerio*) embryos did not result in increased mortality.

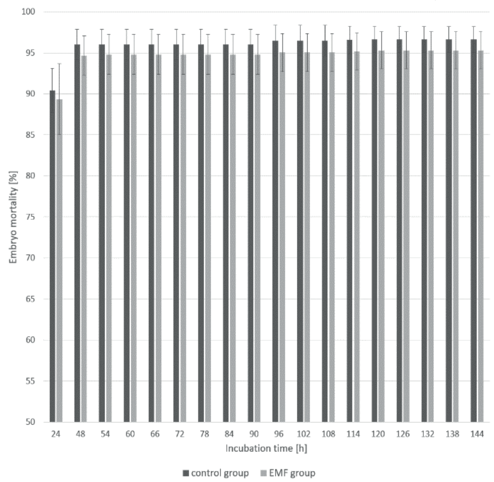

Figure 4. The effect of electromagnetic field exposure on embryo mortality rate of common carp (*Cyprinus carpio*)

In our study, the first hatched larvae were observed after 96 hours of incubation – both in the control and experimental groups. The percentage of hatched larvae at individual time points seemed to be higher in the experimental group (Figure 5), however, the analysis performed showed that the group factor was statistically insignificant ($p = 0.226$). Finally, 38.83% more larvae hatched in the experimental group than in the control group.

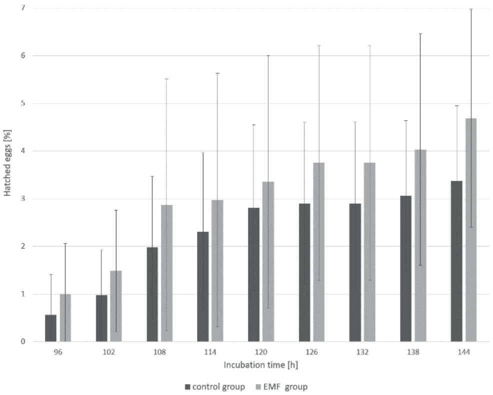

Figure 5. The effect of electromagnetic field exposure on hatching dynamics of common carp (*Cyprinus carpio*)

Lee & Yang (2014) demonstrated that exposure to an electromagnetic field (3.2 kHz; 15-60 μT) significantly accelerated embryonic development of Japanese rice fish (*Oryzias latipes*). Moreover, in the study conducted by Pawlak et al. (2016) a significant acceleration of hatching of chicken (*Gallus gallus domesticus*) exposed to 900 MHz electromagnetic field during incubation was demonstrated. On the other hand, Li et al. (2014) showed that ELF-MF exposure caused significantly decreased hatching rate of zebrafish (*D. rerio*).

In the present study the percentage of deformed larvae among all hatched larvae was higher in the experimental group (Fig. 6), however, these differences were not statistically significant ($p = 0.058$). Similarly, Li et al. (2014) demonstrated that exposure to ELF-MF did not cause increased percentage of malformations in zebrafish (*D. rerio*).

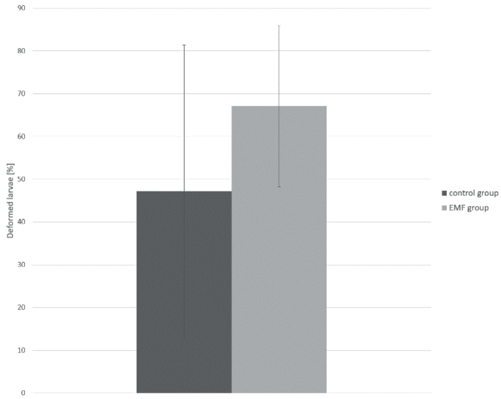

Figure 6. The effect of electromagnetic field exposure on the occurrence of deformations in common carp (*Cyprinus carpio*)

Roundup exposure

There was a statistically significant increase in the percentage of dead eggs (Figure 7) in group R1 after 144, 150 and 168 h of incubation compared to the control value. The percentage of dead eggs was also statistically significantly higher than in the control in group R2 after 24, 114, 120, 126, 132, 138, 144, 150, 156, 162 and 168 h. The proportion of dead eggs was statistically significantly increased in group R3 compared to the control group after 24, 54, 60, 66, 114, 120, 126, 132, 138, 144, 150, 156, 162 and 168 h. There was also a statistically significant reduction in the percentage of dead eggs in group R1 compared to the control value after 78 h of incubation.

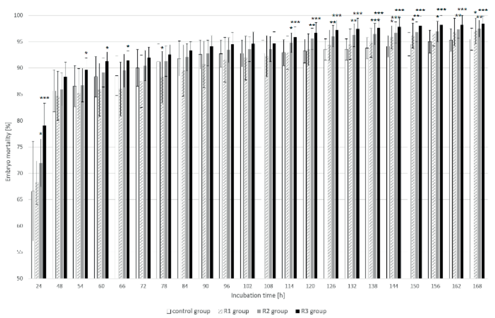

Figure 7. The effect of Roundup exposure on embryo mortality rate of common carp (*Cyprinus carpio*)

Fiorino et al. (2018) observed significantly higher mortality of common carp (*C. carpio*) eggs after 48 hours post fertilization (hpf) at 10 mg/l and after 96 hours at 5, 10, and 50 mg/l; after 120 hpf at 0.005, 5, 10, and 50 mg/l compared to the control. In the case of zebrafish (*D. rerio*) the same authors also noted increased embryo mortality. Significant differences were found at 0.05, 5, 10, and 50 mg/l after 48, 72, and 96 hpf and at 5 and 50 mg/l after 120 hpf. Moreover, Zhang et al. (2021) who studied the effects of glyphosate exposure (72 hours) on zebrafish (*D. rerio*) embryos demonstrated that the survival rate was significantly reduced in comparison to the control in groups exposed to 10, 100, and 700 ng/ml of the herbicide. Similarly, Socha et al. (2021) observed significantly increased mortality rate of common carp (*C. carpio*) embryos exposed to 1 and 10 µl/l of Roundup.

The first hatched larvae were observed after 108 hours of incubation in group R3 (Figure 8).

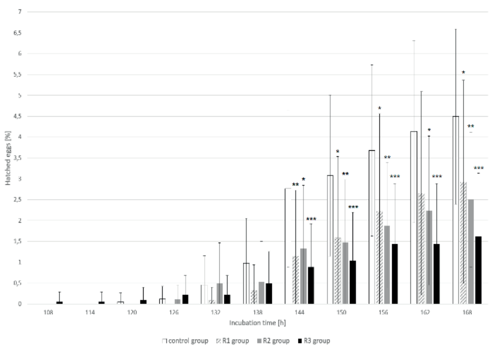

Figure 8. The effect of Roundup exposure on hatching dynamics of common carp (*Cyprinus carpio*)

The next group with hatched larvae were observed was the control group (the first larvae were found after 120 hours of incubation). Hatching dynamics differed significantly depending on the group (p = 0.0007). After 144 hours of incubation, a statistically significant reduction in the percentage of hatched larvae was observed in groups R1, R2 and R3 compared to the control value. A similar situation took place after 150 and 156 hours of incubation. After 162 hours of incubation, the percentage of hatched larvae was significantly lower in group R2 and in group R3, while the value recorded in the case of group R1 did not differ significantly from the control value. After 168 hours of incubation a statistically significant reduction in the percentage of hatched larvae was found in groups R1, R2, and R3 compared to the control group.

It should be emphasised that the statistical analysis did not include the observations made after 108, 114, 120 and 126 hours of incubation due to the lack of sufficient variability within the analysed groups. In the study conducted by Fiorino et al. (2018) common carp (*C. carpio*) eggs exposed to glyphosate exhibited significantly lower hatching rate at 72 (10, and 50 mg/l), 96 (50 mg/l), and 120 hpf (5, 10, and 50 mg/l). On the other hand, low concentration (0.05 mg/l) resulted in significantly higher hatching rate. In the study performed by the same authors, hatching of zebrafish (*D. rerio*) eggs exposed to glyphosate started at 96 hpf in the control group and in the experimental groups treated with the lowest concentrations of glyphosate (0.005 and 0.05 mg/l), while embryos exposed to the same herbicide applied at concentrations of 5, 10, and 50 mg/l began to hatch at 72 hpf. A significantly higher hatching rate was noted at some groups (i.e., 5, 10 and 50 mg/l) at 72 hpf and in all treated groups at 96 hpf. Moreover, Zhang et al. (2021) observed significantly lower hatching rate of zebrafish (*D. rerio*) eggs exposed to 10, 100, and 700 ng/ml of the same chemical. The study conducted by Socha et al. (2021) showed a significant retardation of the hatching rate of common carp (*C. carpio*) in the group treated with 10 µl/l of Roundup.

The percentage of deformed larvae (Figure 9) among all hatched larvae depended on the group factor (p = 0.0000).

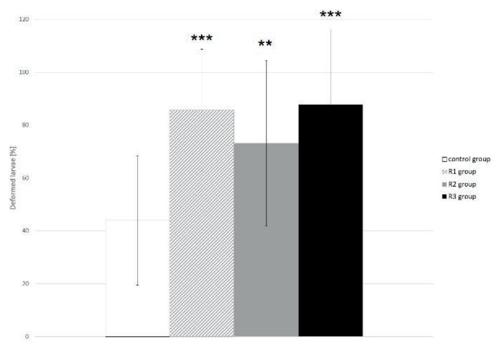

Figure 9. The effect of Roundup exposure on the occurrence of deformations in common carp (*Cyprinus carpio*)

This parameter was statistically significantly higher in groups R1, R2 and R3 compared to the control value.
In the study performed by Fiorino et al. (2018) malformations such as pericardial edema, yolk sac edema, hematoma, and late development were observed in glyphosate-exposed individuals (0.005, 0.05, 5, and 10 mg/l). In case of zebrafish (*D. rerio*) larvae hatched from eggs exposed to the same herbicide exhibited lower percentages of malformations in comparison to common carp (*C. carpio*). According to Zhang et al. (2021) exposure to glyphosate (10, 100, and 700 ng/ml) resulted in significantly increased percentages of deformed zebrafish (*D. rerio*) larvae. Similarly, Socha et al. (2021) revealed that Roundup treatment resulted in significantly increased percentage of deformed common carp (*C. carpio*) larvae.

CONCLUSIONS

The research conducted showed that the deprivation of the geomagnetic field caused hatching acceleration by 12 hours. At the same time in this group a decrease in the percentage of finally hatched larvae was observed, while exposure to the 1800 MHz electromagnetic field resulted in an increase in this parameter (statistically insignificant changes). Exposure of embryos to Roundup caused significantly increased mortality, marked reduction in hatchability, and clear increase in the percentage of deformed larvae. Due to the high embryo mortality detected in this study in all groups (including the control groups), the results should be considered preliminary. Due to high mortality further research is necessary to confirm the observed phenomena.

ACKNOWLEDGEMENTS

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THE PHYSIOLOGICAL EFFECTS OF SOME STRESS INDICATORS IN RAINBOW TROUT RAISED IN DIFFERENT SYSTEMS

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Abstract

Regardless of the accuracy of the technology implementation, the final quality of the fish is also influenced by the conditions to which it is subjected after being extracted from the rearing pond. In the present case study, we proposed an analysis of the physiological reaction of the stock after extraction from the growth ponds. Among others, we were particularly interested in the level of the stress hormone, determined in the samples taken in the study under the conditions of the application of certain stressors. In contrast, comparison of the amount of glycogen led to obtaining very distinctly significant statistical fluctuations (the mean for specimens reared in semi-intensive system was 2.314±0.638 g/100 g and for those grown in intensive system 1.980±0.822 g/100 g). Growth hormone varied between 0.504±0.46 ng/ml in the case of semi-intensive system and 0.694±0.22 ng/ml for intensive system. The values obtained for cortisol showed a significant influence of stress factors on the studied trout. The stress hormone level rises in correlation with both the intensity and type of stressors, starting from the moment the fish are removed from the ponds.

Key words: *growth system, hormones, salmonids, stressors.*

INTRODUCTION

Worldwide, the highest colonization rate for commercial exploitation is observed in rainbow and brown trout. However, the two species have different responses in terms of adaptation capacity and ecological impact (McGlade et al., 2022).

Many environmental factors specific to salmonid growth (temperature, pH, turbidity, toxic substances, diseases, food etc.) are stress generators when their levels exceed normal physiological limits (Schreck & Tort, 2016).

Biodiversity of animal species is crucial for various reasons, and its importance extends across ecological, economic, scientific, cultural, and ethical dimensions (Davidescu et al, 2023).

When stressors act for short periods, the adaptive response of fish is fast, and they have the ability to restore homeostasis. Diametrically opposite, their long-term maintenance (chronic stress) leads to the appearance of negative effects on the immune system, productivity and health of the livestock (Sneddon & Wolfenden, 2016; Simeanu et al., 2022; Popa et al., 2023).

Rising water temperatures (e.g. due to global warming) affect all species, including rainbow trout, which is a cold-water fish. Biochemical studies carried out on specimens grown in warm waters (20-24°C) revealed significant changes in liver metabolites (aminotransferase, lysozyme, total bilirubin, alkaline phosphatase, superoxide dismutase, glutathione peroxidase and malondialdehyde, which did not return to normal values even after passing into water with normal temperature (14°C) (Li et al., 2022).

Different solutions have been tested to prevent the negative effects of heat stress on rainbow trout. For example, a supplement of 5 mg/kg nanoselenium introduced into the food of rainbow trout subjected to heat stress (24°C) significantly increased the activity of liver glutathione peroxidase. Thus, the levels of alanine aminotransferase, aspartate aminotransferase, superoxide dismutase and malondialdehyde were reduced and, on the

other hand, lipid accumulations in the liver decreased and its tissue structure improved (Marin et al., 2020; Li et al., 2022; Simeanu et al., 2022; Surmeli et al., 2023).
In rainbow trout raised under conditions of thermal stress (+24°C), through high-throughput sequencing of the kidney tissue, microRNAs involved in the response of some target genes to thermal stress, including the transformation of proteins in the endoplasmic reticulum, were identified (Ma et al., 2019).
Also in this sense, the idea was launched that long noncoding RNAs (lncRNAs) can be used in the selection of genetic variants of heat-resistant trout, given their essential role in the regulation of heat stress by association with genes involved in immune regulation, apoptosis and signalling pathways of metabolic activity (Zhou et al., 2022).
A common problem in intensive fish farming is poor water quality (especially dissolved oxygen, turbidity, and total dissolved solids), which significantly affects the growth performance of rainbow trout and greatly increases stress indicators (Welker et al., 2018; Usturoi et al., 2023).
Another stressor is the pH value of the water (acid stress), an indicator influenced by acid rain, acid pollutants, acid wastewater, and the application of excessively high densities. Interestingly, the studies highlighted that exposure of rainbow trout to acidic water (pH-5.2) for 4 days led to increases in the activity of glycoproteins, lysozymes, and myeloperoxidase only in diploid specimens, and non-specific immune functions were not affected in triploid fish (Yilmaz et al., 2017).
Evaluation of the effect of some stressful factors (water temperature, handling, and low water level in the ponds on some antioxidant enzymes in rainbow trout revealed a significant increase in glutathione peroxidase and catalase in all analysed situations, glucose 6-phosphate dehydrogenase only at high water temperature, and glutathione reductase in specimens stressed by handling and low water (Oezmen et al., 2007).
The stress generated by exposure to air (3 min) caused an acute response for 24 h post-exposure, which resulted in significant increases in cortisol, lactate, and plasma glucose as an expression of the reactivity of liver microRNAs (Ikert et al., 2021).
In the case of juvenile rainbow trout reared in small volume tanks, the application of isolation stress resulted in increases in plasma cortisol, glucose, and lactate after one hour of treatment. In the same specimens, after two hours of isolation stress, significant increases in the values of the three indicators were observed; however, during this interval, food consumption was also drastically reduced (Pankhurst et al., 2008).
The duration of the pre-sacrifice period is another stressor that correlates with the frequency of the feeding schedule. Studies have shown that feeding every other day and fasting for two days prior to slaughter results in lower cortisol levels and higher triglycerides and liver glycogen levels than daily/4-day feeding followed by fasting for 9 days, an aspect that indicates a reduction in the response to food stress (Bermejo-Poza et al., 2016; Usturoi et al., 2023).
Although salmonids react well to various stress factors, repeated and chronic exposure to such conditions alters physiological processes and metabolism, with effects on growth and development, reproductive function, and immune response (Rousseau & Dufour, 2017).
Most studies have shown that plasma cortisol is the best indicator of acute stress in fishes. In parallel, the researchers believe that it is essential to identify other molecular, biochemical or hormonal markers, which reflect more accurately the state of stress, in order to improve the productivity and quality of the meat obtained in aquaculture (Vijayan et al., 2010).
The current trend in the Romanian population, also observed at the global level, is to eat in the healthiest possible way. In this context, consumers purchase products obtained in the simplest possible way, as close as possible to what many call "natural".
In general, we mention the fact that the area where the trout farms under study are located is one of real and major tourist interest. This shows that the area is frequently visited by a large number of tourists who, in addition to tourist attractions, are also directly interested in the culinary typology of the area.

Trout is the producer of high-quality meat that is appreciated from both a taste and nutritional point of view. In the area from which the trout comes, there are a multitude of such holdings, most of which are semi-intensive and intensive.
Starting from the previously mentioned premises, through the present work, the authors proposed to carry out a case study in which to observe how rainbow trout react, from the point of view of the state of stress, to different growth conditions.

MATERIALS AND METHODS

The present work represents a case study that aims to follow the way rainbow trout react, from the point of view of stress factors, to different growth conditions.
The objectives taken into account were the monitoring of some water quality parameters and the observation of the physiological responses of the trout (glucose, glycogen, growth hormone, cortisol) under the conditions provided by the two growth systems.
The fish were captured randomly from the breeding ponds, with the limitation of stressing them as much as possible. Later, to apply the stress factors, the trout were kept in special ponds.
Water quality was monitored for a week to exclude the existence of significant fluctuations that would disturb the state of stress in the trout. Samples were collected and the following indicators were determined: temperature, dissolved oxygen, and pH value. The research took place at the end of September-2022, because it is an interval in which moderate temperatures and precipitation are recorded, which leads to constant water quality.
The samples were collected by authorized personnel in characteristic containers and were subjected to analysis.
The dosage of the stress hormone was determined under different circumstances for both trout as follows:
- unstressed: taking the samples immediately after extraction from the pond, without applying a stress beforehand;
- stressed "0 h" - sampling after applying stress factors (lack of oxygen, handling, and higher water temperature);
- stressed "1 h" - sampling after applying stress factors (lack of oxygen, handling, higher water temperature) and keeping them on ice (additional stress factor) for a period of one hour.

Biological material
The biological material was rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792), aged 2 years and a summer. The samples were taken from 36 specimens directly from the aforementioned trout farms (18 specimens/farm).

Location of farms
The trout farms studied are located in a mountainous area, in the central-eastern part of Romania, at a close distance from each other, subject to similar pedoclimatic conditions.
This area is characterized by high temperatures in the warm season (25-30°C) and low temperatures during the cold season (up to -25°C). The area is highlighted by the existence of abundant precipitation, especially during the cold season (the snow layer is maintained for up to 120 days in the valley area and up to 160 days on the slopes).
Both holdings were designed outdoors, as there were no closed farms in the area. This results in a potentially major influence of environmental factors on everything that means "development" of livestock.

The growth and nutrition system
The farms, one of which operates in a semi-intensive exploitation regime and the other in an intensive system, are symbolically marked P-si, and P-i.
The semi-intensive farm (P-si) has a production capacity of approximately 3 tons/year and earthen basins (with a gravel hearth).
The water depth is between 0.8 m (at the inlet) and 1.8 m (at the monk).
The ponds were fed with water from a nearby stream, ensuring a constant flow of approximately 60 L/s.
For consumption of trout, granulated combined feeds with 42% P.B. and 4100 kcal/kg E.M. were administered in a single daily portion, representing 0.9% of the fish's mass.

The intensive rainbow trout farm (P-i) was designed for a production of 20 tons/year and consists of concrete basins (including the hearth), with depths between 1.3 m (at the inlet) and 1.9 m (to monk).
The water source is a natural mountain stream that springs from approximately 1.5 km from the holding, which can ensure a flow of approximately 200 L/s.
Feeding was carried out with extruded granulated feed (44% B.W. and 4200 kcal/kg E.M.) administered in two sessions/day, in a daily amount that represented 1.1% of the fish mass.

Water quality determination

It was highlighted by monitoring three main parameters, known to be potential generators of stress in salmonids, namely: temperature, amount of dissolved oxygen and pH value (Păsărin, 2007). The determinations were made three times a day, at 24^{00}, 8^{00}, and 16^{00}, for seven days, and the average values for the two holdings were then calculated. Probing depth was established in the comfort zone of the fish, approximately 90 cm from the pond earth.
Water characteristics may change rapidly as a result of chemical, physical or biological processes in the water sample. For this reason, whenever applicable, it is advisable to measure the parameters value immediately at the sampling point. The water sample is placed in a 100-150 ml chemical beaker. The electrodes are inserted into it in such a way that they are completely inserted into the water. After 0.5-1 min, the values are read.
The temperature was recorded with the help of a professional digital thermometer used in aquaculture-type TFA-Dostmann, produced by the Roth company.
The determination of dissolved oxygen was carried out with the help of TMT-DO-5512SD.
The pH value was determined using the WTW ProfiLine pH 3310 portable pH meter, produced by the Toth company.

Biochemical and immunological determinations

They were performed in an accredited laboratory by personnel authorized to perform these tests. Biochemical analyses were performed using an ILab Taurus analyzer and laboratory instrumentation. All determinations were performed in accordance with the methodology and legislation. Venous blood was collected in an amount of approximately 0.5 mL in collection containers (vacutainer without anticoagulant with separating gel). Afterwards, the serum was separated by centrifugation, the sample being stable for 7 days at 2-8°C.

Blood glucose measurement

Determination of glucose in the serum was performed using the GOD-PAP method at a wavelength of 510 nm on the ILab Tautus automatic biochemistry analyzer. The measurement range was between -35,000 ÷ +35,000 mAbs/10. To check the performance parameters, the "ready to use" the quantILab glucose (oxidase) mono reagent was used, kept in stable conditions (2-8°C), and within the validity period. The reagent was chosen because there is an adaptable work protocol provided by the manufacturer for the equipment used in this study. The reference material (calibrator) used was Randox calibration serum (code CAL2351). The traceability certificate of the calibrator specifies the traceability of the glucose value to the certified reference material and the total measurement uncertainty (Strath et al., 2013; World Health Organization, 2002).
The reagents used to determine the amount of glucose, code GL3815, lot I0822263, are dedicated to the equipment used and are within their validity period according to the quality certificate. The reagents were stable at 2-8°C until the expiration date was recorded on the bottle and 28 days onboard the analyzer. ILab Assayed Multiser control sera (with the matrix and measured values) were used. In medical laboratories for analytical biochemical determinations, only two concentration levels are generally used for control sera that are relevant in clinical decisions.
Way of working. The GOD/PAP method was developed for quantitative determination of glucose and other biological liquids. The GOD/PAP method has the following measurement principle: the glucose in the sample to be analysed is hydrolysed in the presence of glucose oxidase (GOD, component enzyme in reagent R1) with the formation of

gluconic acid and hydrogen peroxide (World Health Organization - WHO, 2002). This, in the presence of phenol and aminophenazone and under the action of peroxidase (POD, the 2nd enzyme in reagent R1) forms a red colored compound (quinoneimine) (Burtis et al., 2005; Bartiam & Trinder, 1972). The optical density of the colored compound formed is directly proportional to the amount of glucose in the sample and is measured at 510 nm. The GOD/PAP method does not require special environmental conditions. The reactions during the measurement take place on automatic equipment equipped with a thermoregulation system. The ambient working conditions are: temperature of 21±2°C and humidity 40-60% (Bartiam & Trinder, 1972).

Quantitative determination of glycogen

Glycogens are the main storage form of carbohydrates in the animal body. Glycogen is present in all animal organisms at different stages of development. In higher animals, the liver is the organ richest in glycogen. In smaller quantities, this polyglucoid is also found in muscles, the brain, etc. (World Health Organization - WHO, 2002).

Animal tissue undergoes heat desmolysis in a strongly alkaline solution. The glycogen contained in the desmolyzate, in the presence of concentrated sulphuric acid, is split into glucose, which is dehydrated with the formation of hydroxymethyl-5-furfural (Burtis et al., 2005).

Hydroxymethyl-5-furfural condenses with anthrone (9,10-dihydro-9-oxoanthracene), resulting in a blue-green coloured compound, the intensity of which was directly proportional to the glucose concentration obtained following the hydrolysis of the glycogen contained in the analysed tissue. Condensation is carried out between the aldehyde group of 5-hydroxymethylfurfural and the active methylene group from the anthrone molecule (Burtis et al., 2005).

Reagents used were: 30% potassium hydroxide solution; 95% sulphuric acid solution. It was added 100 ml of concentrated sulphuric acid to 5 ml of distilled water and cool. For obtaining 0.2% anthrone solution in 95% sulphuric acid it was dissolved 0.2 g of anthrone in 100 ml of 95% sulphuric acid. The reagent is extremely unstable and therefore it will be prepared no more than 1 hour before the determination, using it only on that day (Young & Friedman, 2001).

The work protocol starts with introductions of 3 ml of potassium hydroxide solution (reagent 1) in a 20 mm ×150 mm heat-resistant glass test tube and closing it with a rubber stopper. A quantity of 0.1-0.5 g of tissue to be analysed (liver, muscle, brain, etc.) cut into small pieces is then weighed on the analytical balance and introduced into the potassium hydroxide solution in the test tube. The stopper was removed and the test tube was placed in a boiling water bath for 20 min. After cooling with tap water, the contents of the test tube were transferred to a 50-100 ml volumetric flask, washing several times with 4-5 ml of distilled water; filled to the mark and shacked vigorously (Schumann et al., 2010).

From the glycogen solution thus obtained, it measured 0.5-2.5 ml in a 30 x 200 mm heat-resistant glass test tube and top up the volume to 2.5 ml with distilled water, if is necessary. Distilled water (2.5 ml) was used to control the reagents in another identical test tube. Both test tubes were placed in an ice-water bath. After cooling, 5 ml of anthrone solution in 95% sulphuric acid (reagent 3) was carefully measured from the burette into each test tube in a thin thread and continuously stirred. The test tubes were covered with a glass pear and placed in a boiling water bath for 10 min. They were then cooled in an ice water bath and immediately the extinction was read on a spectrophotometer at a wavelength, λ = 620 nm (Young & Friedman, 2001; Schumann et al., 2010).

After the extinction value obtained, the amount of glycogen (mg) in the volume of the solution used is found on the standard curve. The glycogen content, expressed in mg %, in the studied tissue is then calculated according to the formula:

$$X = \frac{a \cdot V_1 \cdot 100}{V_2 \cdot P} \text{ (mg \%)}, \qquad (1)$$

were:

a - the amount of glycogen, in mg, found on the calibration curve;

V1 - the volume (ml) to which the desmolyzate obtained after the treatment with potassium hydroxide was diluted;

V2 - the volume of the glycogen solution (ml) taken for the lane reaction;
P - the weight of the analysed tissue (g);
100 - conversion factor in percentage.
To construct the standard curve, a glycogen solution was used, which was prepared by dissolving 40 mg of glycogen in a 500 ml volumetric flask in hot distilled water. After cooling the solution to room temperature, it was made up to the mark with distilled water and shaken. This solution contains 0.08 mg of glycogen in one millilitre. From the obtained solution, prepare a series of samples for drawing the standard curve by pipetting the volumes of the solution.
It is recommended that the addition of 0.2% anthrone solution in 95% sulphuric acid be performed at certain intervals (e.g., 2 min) from one test tube to the next. This time interval will also be respected when reading extinctions after cooling the samples to room temperature. The extinction of the cooled samples was measured on a spectrophotometer at a wavelength λ of 620 nm. The standard curve is drawn by writing the glycogen concentration (mg) on the abscissa and the extinction values E on the ordinate (Schumann et al., 2010).

Cortisol dosage

The cortisol (antigen) in the sample competes with peroxidase (enzymatically labelled antigen) to bind a limited number of anti-cortisol antibodies in the microplates (solid phase). After incubation, bound/free separation was accomplished using a simple solid phase wash. The enzyme substrate (H2O2) and TMB substrate were added. After an appropriate time for maximum colour development, the enzymatic reaction was stopped and the absorbance was determined. Cortisol concentration in the samples was calculated based on a standard series. The intensity of the colour is inversely proportional to the concentration of cortisol in the sample (Gornall et al., 1949).
The kit determination included: micro titration plate with 96 wells; standard reference cortisol set, containing 0, 10, 50, 150, 500 ng/ml; control reagent; conjugated reagent, 13 ml; TMB (One-Step) reagent, 11 ml; stop solution (1N HCl), 11 ml.
To determine cortisol levels, the samples were processed in duplicate. It was prepared two wells for each of the five points of the standard curve (S0-S4), two for each sample and one for Blank (Toni et al., 2019).

Growth hormone dosage

Growth hormone (GH) is a polypeptide that is secreted by the anterior pituitary gland. It promotes protein conservation and is involved in a wide range of protein synthesis mechanisms. GH also improves glucose transport and facilitates glycogen storage.
The test system used a solid-phase anti-GH polyclonal antibody (microtiter wells) and a mouse anti-ferritin monoclonal antibody in an antibody-enzyme (horseradish peroxidase) conjugate solution. The sample was allowed to react simultaneously with the antibodies, resulting in the formation of GH molecules between the solid phase and the enzyme antibodies. After 60 min incubation at room temperature, the wells were washed with water to remove unbound labelled antibodies. TMB solution of TMB is added and incubated for 20 min, resulting in the appearance of a blue colour. Colour development was stopped by the addition of 2N HCl and the colour changed to yellow, which was measured spectrophotometrically at 450 nm. The GH concentration is directly proportional to the colour intensity of the test sample (Young, 2000; Toni et al., 2019).
Determinations were made with the HGH ELIZA kit, Lot 5811A, Ref. DKO 050, produced by DIA METRA, Italy.
The determination kit included: micro titration plate with 96 wells; standard reference set, containing 0, 2.5, 5, 10, 25, and 50 ng/ml of GH, ready for use; conjugated reagent, 13 ml; TMB (One-Step) reagent, 11 ml; stop solution (1N HCl), 11 ml.
The working protocol included the following steps:

- set the desired number of covered wells in the work support;
- pipette 50 µl of the standard and control solution into the appropriate wells;
- distribute 100 µl of conjugated reagent in each well;

- homogenize well for 30 seconds (complete homogenization is very important at this stage);
- incubate at room temperature (18-25°C) for 60 minutes;
- throw the incubation mixture contained in the plate into a waste container;
- rinse and shake the micro titration wells 5 times with distilled or deionized water;
- suddenly tap the work plate on a support of paper or absorbent towels to remove all residual water drops;
- distribute 100 µl of TMB reagent in each well. Shake gently for 10 seconds;
- incubate at room temperature for 20 minutes;
- stop the reaction by adding 100 µl of stop solution to each well;
- shake gently for 30 seconds (it is important to be sure that all blue wells turn completely yellow);
- the samples are read at the optical density of 450 nm, with a microtiter plate reader, in a maximum of 30 minutes.

Data processing

The experimental data were processed using the calculation algorithms in Microsoft Excel, and the statistical interpretation was based on the SPSS Statistics 21.

RESULTS AND DISCUSSIONS

Environmental factors

Rainbow trout is one of the less pretentious species in terms of the physicochemical characteristics of water compared to other salmonids. This species tolerates turbid waters quite easily, but only for short periods of time, also during the hottest seasons, but with high flows (approx. 1 L/min/kg fish) and relatively rich in dissolved oxygen (over 6 mg O_2/L) (Schreck et al., 2016; Sneddon & Wolfenden, 2016).

In order to accurately determine the influencing factors on the health status of the analysed populations, it was monitored a series of growth parameters in the two studied trout farms. Therefore, the technical parameters applied at the farm level, the water quality in the rearing tanks, and the characteristics of the combined feed administered for trout feeding were taken into consideration.

The technical parameters studied included water flow rate, population density, and trout weight.

It was observed that a significantly higher water flow rate was used in P-i, with a rate of 200 l/sec, compared to only 60 l/sec in P-si. This parameter was evidently influenced by precipitation; however, during the analysis period, it did not represent a significant factor.

A similar remark can be made in the case of the applied densities, which are 25 and 75 head/m^2 for P-si and P-i, respectively.

Differences also existed in the body weight at the time of the study. Thus, the average value obtained for P-si was 262.84±5.12 g, while for P-i 274.36±5.23 g (Table 1). Within the batches, the character was homogeneous, the coefficient of variation indicating very small values.

In the trout farms, the water flow was ensured to be 60 L/sec at P-si and 200 L/sec at P-i.

This fish feeds efficiently at water temperatures between 15°C and 19°C but stops feeding above 23°C. In deep water, it is a feared predator (Păsărin, 2007).

Studies have shown that rainbow trout adapt best to intensive growth in farms designed according to modern principles; in some specialized lines, reproduction can be induced in all seasons of the year (Pankhurst et al. 2008; Li et al., 2022).

Water temperature plays an important role in the growth of salmonids because it influences the feeding and body temperature of trout (McGlade et al., 2022). Optimal feeding temperatures and a high digestibility for rainbow trout are at 15-19ºC (Yilmaz et. al., 2017). The brown trout is particularly demanding of water temperature, which must not fall below 4°C or exceed 15°C (when feeding stops). Feeding activity is more intense between 12°C and 14°C (Păsărin, 2007).

Water quality was analysed using three indicators: temperature, pH, and dissolved oxygen.

Within the two rainbow trout farms it was found that the temperature values had variations, but insignificant, the average being 11.24±0.76 at P-si and 10.14±0.40 at P-i (Table 1). In both cases, there were no drastic increases or decreases in parameter values, which could lead to trout stress.

The pH value in the water ponds has fluctuated around 7.5, with an average 7.28±0.14 of P-si and 7.65±0.22 at P-i. In this context, according to STAS 4706/1988, the water can be classified as II quality level. For salmonids, an optimal pH must be between 7.5 and 8.5 (Li et al., 2022).

The amount of dissolved oxygen determined in the water of studied farms was 8.46±0.22 mg/L P-si and 9.82±0.18 mg/L at P-i, and no significant fluctuations were recorded. Water who has a content of dissolved oxygen over 9 mg/L, and temperatures of 18-19°C, is considered appropriate for trout rearing. Dissolved oxygen concentration has been identified as a critical factor for the survival of salmonids in all phases of development, from the fry to the reproductive stage. The concentration of dissolved oxygen in water is inversely proportional to water temperature (Păsărin, 2007). Salmonids can live in water with an oxygen content of 9-10 mg/L (Schreck & Tort, 2016).

The administered combined feeds were evaluated in terms of protein content, metabolizable energy, number of taints, and amount administered.

The protein level of the recipe administered at P-si was 41%, whereas that at P-i was 43%. Differences also existed in the case of metabolizable energy, for which the values were 4100 kcal/kg (P-si) and 4300 kcal/kg (P-i).

In both fish farms, three daily feedings were administered, the difference being that in P-si, the amount of combined feed represented 1.1% of the weight of the fish, while in P-i, it represented 1.2% of its weight (Table 1).

Table 1. Experimental factors applied to 2.5 years old rainbow trout

Specification		Semi-intensive system (P-si)	Intensiv (P-i)
The technical parameters	Water flow rate (L/s)	60 L/sec	200 L/sec
	Density	25 heads/m^2	75 heads /m^2
	Rainbow trout weight (g) (average values)	262.84±5.12	274.36±5.23
Water quality (average values)	Water temperature (°C) (average values)	11.24±0.76	10.14±0.40
	Dissolved oxygen (mg/L - average values)	8.46±0.22	9.82±0.18
	pH value (average values)	7.28±0.14	7.65±0.22
Combined feeds administered	Protein content (%)	41	43
	Metabolizable energy (kcal/kg)	4100	4300
	Daily feed intake	3	3
	Proportion of combined feed (% of fish weight)	1.1	1.2

Blood glucose level

Because the concentration of glucose in the blood is an indicator that can be correlated with the stress hormone, it was dosed in the case of the samples taken in this study. In the case of the batch related in semi-intensive P-si system, average values of the parameter of 20.78±1.44 mg/dL were obtained. The minimum value was 19.36 mg/dL, while the maximum value was 22.28 mg/dL. For the lot raised in the intensive system, P-i, the minimum value determined was 18.82 mg/dL and the maximum value was 23.40 mg/dL. The calculation of the mean value indicated a result of 21.10±2.24 mg/dL, which was within the confidence interval of the mean. No statistically significant differences were found between the averages of the two batches (Table 2).

In fish, glucose provides most of the energy consumed during swimming. Normal blood glucose values in salmonids are between 28.41 - 64.00 mg/dL (Barton, 2000). Some factors can indirectly alter blood glucose levels. Some studies suggest that "*growth history, including nutritional status, may affect stress response and glucose release*" (Krasnov et al., 2001). This statement is also supported by other authors who found that blood glucose results must be interpreted considering extrinsic factors because they can affect the glycogen reserve in the liver. This category includes diet, age, time since last feeding, season, and so on (Shanghavi & Weber, 1999).

Nutrition is also an important factor that influences blood glucose levels. Thus, the concentration varies between species and depends on the developmental stage (Jentoft et al., 2005). The intake of diets with different lipid and protein contents results in distinct blood glucose levels (Cheng, 2006).

Under conditions of stress, fish quickly consume glucose because the main function of the central nervous system is to maintain homeostasis; thus, no significant change in blood glucose is observed. However, it is possible that fish exposed to chronic stress may deplete the substrate, leading to a decrease in blood glucose levels (Ruibal et al., 2002).

The research in this paper highlighted average values of the parameter, of 20.78±1.44 mg/dL for P-si and 21.10±2.24 mg/dL in the case of P-i, lower than those stated in the specialized literature (Gilmour et al., 2012).

According to other studies, this phenomenon may be due to the poor quality of the water (in both holdings it fell to II quality), stressful factors (the capture of the studied specimens was done in the cold season), the quality of the feed administered, and the density practiced (Marcos et al., 2014).

The individual values obtained from the biochemical analyses showed variations in blood sugar levels from one individual to another. This aspect is due to the fact that glucose shows variations depending on sex, diet, stress conditions, etc. (Krasnov et al., 2001; Jentoft et al., 2005).

Table 2. Results on glucose analysis in 2.5 years old rainbow trout

Growth system	N	Average values $\overline{X} \pm s_{\bar{x}}$ (mg/dL)	The confidence interval of the mean (95%)		Min. (mg/dL)	Max. (mg/dL)
			Lower limit	Upper limit		
P-si	18	20.78±1.44	19.30	23.10	19.36	22.28
P-i	18	21.10±2.24	19.10	22.90	18.82	23.40
The statistical significance			P-si vs. P-i = n.s.; F=0.013, p=0.911, p> 0.05			

*significant differences between means for 0.01 < p < 0.05.
**distinguished significant differences between means for 0.001 < p < 0.01.
***highly significant differences between means for p < 0.001.

Hepatic glycogen

Hepatic glycogen is a parameter that is significantly influenced by the effort made by the trout and can be correlated with stress hormones. In the case of the P-si group, the determined amount of muscle glycogen was at an average level of 2.314±0.638 g/100 g, the value being higher than that determined for P-i, which was 1.980±0.822 g/100 g.

For P-si, the minimum recorded value of the indicator was 1.345 g/100 g, whereas the maximum was 3.796 g/100 g. In comparison, the minimum value determined for P-i was 1.098 g/100 g, with a maximum of 3.248 g/ 100 g. These values generated significant statistical differences among the studied lots (Table 3).

When interpreting the data, the conditions under which the trout studied were kept were taken into account, as glycogen content can reflect biochemical adaptations to any environmental stress (Figueroa et al., 2000). Of these, pH, oxygen and salinity levels, as well as prolonged physical activity, directly affect glycogen reserves (Winter et al., 2005; Usturoi et al., 2009).

Referring to other bibliographic sources, the amount of liver glycogen is quite low, which is explained by intense physical activity from the moment of capture (Lopez-Patino et al., 2014). Under hypoxic conditions, liver glycogen is mobilized to support the white muscle, suggesting that this is a biochemical strategy used as a response to such stress (Daskalova, 2019).

Table 3. Results regarding hepatic glycogen in 2.5 years old rainbow trout

Growth system		N	Average values $\overline{X} \pm s_{\bar{x}}$ (g/100 g)	The confidence interval of the mean (95%)		Min. (g/100 g)	Max. (g/100 g)
				Lower limit	Upper limit		
Hepatic glycogen	P-si	18	2.314±0.638	1.864	2.878	1.345	3.796
	P-i	18	1.980±0.822	1.534	2.444	1.098	3.248
The statistical significance				P-si vs. P-i=***.; F=48.006, p=0.001, p < 0.05			

*significant differences between means for 0.001 < p < 0.01.
**distinguished significant differences between means for 0.001 < p < 0.01.
***highly significant differences between means for p < 0.001.

Determination of hepatic glycogen content is of particular importance for the investigation of the physiological and pathological states of the animal body as well as for the investigation of the influence of certain factors on carbohydrate metabolism (Viant et al., 2003).
The fluctuation of the results indicates that the main causative factors are different growing conditions. A discrepancy can be observed regarding the liver glycogen reserve in trout specimens raised in a semi-intensive system compared to those raised in an intensive system. From a nutritional perspective, elevated blood glucose levels in trout may be attributed to their energy needs, given that trout are predatory creatures expending significant energy in pursuit of prey (Zhou et al., 2022).

Growth hormone

In the case of fish, growth hormone participates in almost all important physiological processes in the body. Specifically, it is involved in the metabolism of proteins and carbohydrates, growth of the skeleton and soft tissues, reproduction, and functioning of the immune system.
The average level of growth hormone in the specimens raised in the semi-intensive system had a value of 0.504±0.46 ng/ml, slightly lower than the 0.694±0.22 ng/ml, as recorded in the specimens raised in the intensive system. The minimum values recorded for P-si and P-i were 0.06 and 0.35 ng/ml, respectively, while the maximum values were at the level of 0.35 ng/ml for P-si and 0.98 ng/ml in the case of P-i (Table 4). However, no statistically significant differences were observed between the two batches studied, the data being similar to from the specialized literature (Jentoft et al., 2005; Ge et al., 2021)
The growth hormone is a pluripotent hormone produced by the pituitary gland and is secreted in response to exercise, stress, deep sleep, hypoglycemia, and insulin. If growth hormone is secreted deficiently or excessively in the first stages of growth, dwarfism and gigantism will appear respectively. Over the past two decades, many aspects of growth hormone physiology have been the subject of intense research in fish, particularly in salmonids, cyprinids, and sparids (Uiuiu et al., 2021; Morro et al., 2021).
Recent studies have shown that growth hormone affects several aspects of behaviour such as appetite, foraging, aggression and predator avoidance, with the finality of these changes having ecological consequences (Remo et al., 2017; Breves et al., 2020).

Table 4. Results of the analysis of growth hormone in 2.5 years old rainbow trout

Growth system	N	Average values $\overline{X} \pm s_{\bar{x}}$ (ng/ml)	The confidence interval of the mean (95%)		Min. (ng/ml)	Max. (ng/ml)
			Lower limit	Upper limit		
P-si	18	0.504±0.46	0.34	0.75	0.06	1.04
P-i	18	0.694±0.22	0.59	0.80	0.35	0.98
The statistical significance			P-si vs. P-i = n.s.; F=2.859, p=0.100, p > 0.05			

*significant differences between means for 0.01 < p < 0.05.
**distinguished significant differences between means for 0.001 < p < 0.01.
***highly significant differences between means for p < 0.001.

Cortisol

Cortisol is the most active and abundant corticosteroid in fish blood, and its structure has been highly conserved in all vertebrate species in which it is found (Sloman et al., 2001).
The main targets of action of cortisol are the gills, intestine, and liver; they reflect the main adaptive functions of cortisol identified thus far: osmoregularity and maintaining a balanced energy metabolism (Overli et al., 2002).
Consistent with other studies (Best & Glimour, 2022), basal cortisol levels in unstressed salmonids ranged from to 0-5 ng/ml, but acute stress (handling or one hour of confinement) caused a temporary increase in cortisol levels, ng/mL, in the range of 4-20 ng/ml, with a return to basal level in 24-48 hours.
It is recommended that repeated measurements be made during or after acute exposure of the animal, and during chronic experiments, sampling should not be very frequent, as their handling may affect future measurements (Culbert & Glimour, 2016).
The degree of increase in cortisol levels in response to acute stress is also related to the trout species studied. Chronic stress (prolonged labor or crowding) results in an increase in

cortisol levels of approximately 10 ng/ml^{-1}, and blood cortisol levels remain elevated for up to 4 weeks before acclimation (Lequin, 2005; Pfalzgraff et al., 2021).

In specialized literature, pre- and post-stress cortisol variations are presented in two rainbow species of interest, *Oncorhynchus mykiss* and *Salvelinus fontinalis*. After manipulation and isolation, rainbow trout recorded pre-stress values of 77 nmol/l and post-stress values of 698 nmol/l, which was 19 nmol/l in the initial phase but increased to 242 nmol/l after the action of the stress factor (Sepahi et al., 2013; Mota et al., 2017).

In the case of trout studied, cortisol dosing was performed at three distinct intervals:

- unstressed: taking the samples immediately after extraction from the pond, without applying a stress beforehand;
- stressed "0 h" - sampling after applying stress factors (lack of oxygen, handling, and higher water temperature);
- stressed "1 h" - sampling after applying stress factors (lack of oxygen, handling, higher water temperature) and keeping them on ice (additional stress factor) for a period of one hour.

In individuals from the unstressed trout group, an average cortisol value of 86.56±7.34 µg/dL was recorded in the case of P-si, and in those from P-i of 80.28±3.14 µg/dL. The quantitative differences between the two fish batches were statistically significant.

Following the action of the stress factors, the analysed blood samples recorded much higher cortisol values, both in the "0 h" stressed individuals (163.62 ± 17.96 µg/dL in P-si, and 159.87 ± 14.36 µg/dL in P-i), as well as chosen for the "1 h" stressed samples (295.62 ± 14.34 µg/dl in P-si, and 298.12 ± 8.18 µg/dl in P-i). It is mentioned that, for both types of applied stress, the responses of the two species of trout were remarkably similar, the statement being confirmed by the lack of statistical differences (Table 5).

Table 5. Results regarding cortisol analysis in 2.5 years old rainbow trout

Parameter/ Growth system		N	Average values $\overline{X} \pm s_{\bar{x}}$ (µg/dl)	The confidence interval of the mean (95%) Lower limit	Upper limit	Min. (µg/dl)	Max. (µg/dl)
Cortisol in rainbow trout non-stressed	P-si	18	86.56±7.34	82.84	91.64	75.26	95.48
	P-i	18	80.28±3.14	78.96	82.58	77.46	87.98
The statistical significance	P-si vs. P-i= ***; F=10.836, p=0.002, p< 0.05						
Cortisol in stressed rainbow trout at "0h""	P-si	18	163.62±17.96	154.70	174.24	129.82	187.30
	P-i	18	159.87±14.36	153.45	167.80	133.68	180.42
The statistical significance	P-si vs. Pi= n.s.; F=0.469, p=0.498, p> 0.05						
Cortisol in stressed rainbow trout at "1h"	P-si	18	295.62±14.34	288.04	302.26	264.98	312.66
	P-i	18	298.12±8.18	294.02	302.68	286.04	314.98
The statistical significance	P-si vs. P-i = n.s.; F=0.396, p=0.533, p> 0.05						

*significant differences between means for 0.01 < p < 0.05.
**distinguished significant differences between means for 0.001 < p < 0.01.
***highly significant differences between means for p < 0.001.

CONCLUSIONS

Water flows and densities were within specific norms for both intensive and semi intensive systems.

Water quality parameters were similar across both systems, with values within acceptable limits.

Differences in administered feed, including protein and metabolizable energy levels, were noted but supported technologically in both variants.

Blood glucose and liver glycogen levels showed slight variations between rainbow trouts rearing in intensive and semi intensive systems.

Growth hormone levels were higher in intensive system, but still within acceptable ranges for both systems.

Cortisol levels fluctuated, indicating stress responses, with slightly higher values in semi intensive system.

Overall, no significant differences were observed compared to specialized literature, highlighting the existence of major stress factors post-fishing.

Growth system variations did not significantly influence studied parameters.

Better adaptability of specimens in the intensive system was noted, likely due to additional feeding and handling practices.

The study suggests that exploiting salmonids in systems closer to natural conditions may not guarantee superior results compared to intensive systems.

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HYDROBIOLOGICAL MONITORING OF TWO RIVERS FROM THE MARITSA RIVER BASIN BASED ON A BIOLOGICAL QUALITY ELEMENT MACROZOOBENTHOS

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Abstract

In 2023, an ecological assessment of the state of two rivers, part of the Maritsa river basin in Bulgaria, was carried out. For the study, benthic macroinvertebrate organisms (macrozoobenthos) were collected during the spring season from the Luda Yana River in the area of the village of Popintsi (Panagyurishte Municipality, Pazardzhik Region) and from the Chepelarska River in the area of Katunitsa village (Sadovo Municipality, Plovdiv Region), designated as biotopes. 512 and 712 specimens of benthic macroinvertebrates were collected from the Popintsi biotope and the Katunitsa biotope, respectively. The macroinvertebrate taxa found from the Popintsi biotope are belonging to 12 orders: Amphipoda, Annelida, Coleoptera, Diptera, Ephemeroptera, Gastropoda, Hemiptera, Lepidoptera, Lumbriculida, Odonata, Plecoptera, Trichoptera. Macroinvertebrates from the Katunitsa biotope are belonging to 11 orders: Amphipoda, Annelida, Diptera, Ephemeroptera, Gastropoda, Hemiptera, Lepidoptera, Lumbriculida, Odonata, Plecoptera, Trichoptera.

Key words*: benthic macroinvertebrates, Bulgaria, Chepelarska River, ecological assessment, Luda Yana River.*

INTRODUCTION

The Maritsa River rises from Rila Mountain and flows into the Aegean Sea, flowing through the territory of three countries. On the Bulgarian territory, the river has a length of 322 km and a catchment area of 21.084 km^2. The Luda Yana River (74 km) and the Chepelarska River (86 km) are one of the largest tributaries of the Maritsa River (Kiradzhiev, 2013). Both rivers are part of the East Aegean region in Bulgaria. The Luda Yana and Chepelarska Rivers fall into Ecoregion 7 Eastern Balkans. According to the river typology, the Luda Yana River and the Chepelarska River are R3: Mountain type and R5: Semi-mountain type (Belkinova et al., 2013; East Aegean River Basin Directorate, 2018). The rivers falling into the East Aegean basin are subjected to intense anthropogenic pressure from a number of activities, such as mining, discharge of industrial and domestic wastewater, urbanization, alteration of the river bed, extraction of aggregates, and others (East Aegean River Basin Directorate, 2018). The water state of the Luda Yana River is mainly influenced by the activity of enterprises related to the extraction and processing of ores, tailings ponds, and others (Gartsiyanova et al., 2020), located in its upper course. The water quality of the Chepelarska River has deteriorated mainly in the lower reaches of the river, as a result of the activities of industry, mining, tailings ponds, and others (Municipal environmental protection program of Assenovgrad Municipality, 2018-2027). The use, management, distribution, and assessment of water resources in the East Aegean catchment basin was considered by Nikolova et al. (2010); Kolcheva (2016; 2019; 2020); Kolcheva & Ilcheva (2016). The Water Framework Directive aims to achieve good surface water's ecological and chemical status. To assess the ecological status of the water, three groups of elements are monitored: physicochemical, biological, and hydromorphological (Belkinova et al., 2013; Kolcheva et al., 2023). According to the Water Framework Directive, biological quality elements (BQEs) are leading in the ecological assessment of aquatic ecosystems (Belkinova et al., 2013). Benthic macroinvertebrate organisms (macrozoobenthos) are good bioindicators for assessing the ecological status of river ecosystems (Varadinova et al., 2022). Scientific studies to assess water quality based on BQE macrozoobenthos from the Luda Yana River were carried out by Vidinova et al. (2008) and Georgieva et al. (2014). The water condition of the Luda Yana River was studied by Gartsiyanova et al. (2020); Gartsiyanova (2021); Gartsiyanova et al. (2021); Radeva & Seymenov (2021); Gartsiyanova et al. (2022). There are few studies on the benthic macroinvertebrate fauna from the Chepelarska River (Vidinova et al., 2008; Park et al., 2022; Varadinova et al., 2022).

The aim of the present study is to carry out hydrobiological monitoring of the Luda Yana River and the Chepelarska River (part of the Maritsa River Basin in Bulgaria) based on BQE macrozoobenthos.

MATERIALS AND METHODS

In the spring of 2023, samples of benthic macroinvertebrate organisms were collected to carry out an ecological assessment of the water state of the Luda Yana River and the Chepelarska River. The sampling was carried out in the Popintsi village, along the Luda Yana River, and in the Katunitsa village, along the Chepelarska River (Figure 1).

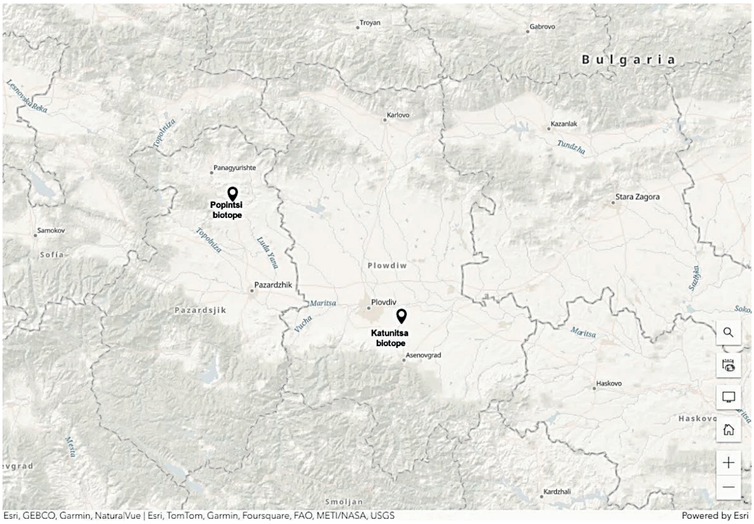

Figure 1. Investigated biotopes from the Luda Yana River and the Chepelarska River (https://www.esri.com/en-us/arcgis/products/arcgis-online/overview)

Sampling of macroinvertebrate organisms is according to Cheshmedjiev et al. (2011); EN ISO 10870:2012; EN 16150:2012; Ordinance H-4 of 14.09.2012; Belkinova et al. (2013). The collected samples were stored in 70% ethyl alcohol for further processing in laboratory conditions. The samples were processed in the laboratory of the Department of Agroecology and Environmental Protection at the Agricultural University - Plovdiv.

Based on an established methodology (Ordinance H-4 of 14.09.2012; Belkinova et al., 2013; and others), the following metrics were calculated: 1) Taxonomic composition of the benthic invertebrate fauna (total number of taxa; EPT - number of Ephemeroptera, Plecoptera and Trichoptera taxa); 2) Abundance of the benthic macroinvertebrate fauna (% Oligochaeta & Diptera, % Filtering feeders, % EPT taxa and the German trophic index RETI); 3) Saprobity; 4) Indices for species diversity (Species richness index of Margalef (Dmg); Shannon-Weaver species diversity index (H'); Pielou's evenness index

(E); Simpson's dominance index (C) and 5) Integrated indices for the benthic macroinvertebrate fauna (Adapted Biotic Index by Flanagan & Toner, 1972; modified by Clabby & Bowman, 1979; Clabby, 1982).

RESULTS AND DISCUSSIONS

Taxonomic composition of the benthic invertebrate fauna

The hydrobiological monitoring was carried out according to the methodology for biomonitoring approved for the European Union and Bulgaria, based on a biological quality element macrozoobenthos. The taxonomic composition of the macrozoobenthos from the two biotopes was determined. In the Popintsi biotope, 23 taxa (including Nematoda) were established, represented by 512 specimens. In the Katunitsa biotope, 20 taxa were found, with a total number of 712 specimens (Table 1). Based on the "total number of taxa", the ecological status of the Luda Yana River (Popintsi) and the Chepelarska River (Katunitsa) was defined as "high" (16+).

Table 1. Taxonomic composition of macroinvertebrate organisms from Popintsi biotope (Luda Yana River) and Katunitsa biotope (Chepelarska River)

TAXON	GENUS	FAMILY	ORDER	BIOTOPE
***Acentria ephemerella* (Olivier, 1791) [syn. *Acentropus niveus* (Olivier)]**	*Acentria* Stephens, 1829	Crambidae	Lepidoptera	Katunitsa
***Agapetus* sp.**	*Agapetus* Curtis, 1834	Glossosomatidae	Trichoptera	Popintsi; Katunitsa
***Anabolia* sp.**	*Anabolia* Stephens, 1837	Limnephilidae	Trichoptera	Katunitsa
***Baetis* sp.**	*Baetis* Leach, 1815	Baetidae	Ephemeroptera	Katunitsa
***Caenis horaria* (Linnaeus, 1758)**	*Caenis* Stephens, 1835	Caenidae	Ephemeroptera	Popintsi
***Cataclysta lemnata* Linnaeus, 1758**	*Cataclysta* Hübner	Crambidae	Lepidoptera	Popintsi
***Centroptilum luteolum* Müller, 1776**	*Centroptilum* Eaton, 1869	Baetidae	Ephemeroptera	Katunitsa
***Ecdyonurus* sp.**	*Ecdyonurus* Eaton, 1868	Heptageniidae	Ephemeroptera	Popintsi; Katunitsa
***Enallagma cyathigerum* Charpentier, 1840**	*Enallagma* Selys, 1876	Coenagrionidae	Odonata	Popintsi; Katunitsa
***Ephemerella ignita* Poda, 1761**	*Serratella* Edmunds, 1959	Ephemerellidae	Ephemeroptera	Popintsi; Katunitsa
***Galba truncatula* (O.F. Müller, 1774)**	*Galba* Schrank, 1803	Lymnaeidae	Gastropoda	Popintsi
***Gammarus* sp.**	*Gammarus* Fabricius, 1775	Gammaridae	Amphipoda	Popintsi; Katunitsa
***Gomphus* sp.**	*Gomphus* Leach, 1815	Gomphidae	Odonata	Popintsi; Katunitsa
***Habrophlebia* sp.**	*Habrophlebia* Eaton, 1881	Leptophlebiidae	Ephemeroptera	Katunitsa
***Halesus* sp.**	*Halesus* Stephens, 1836	Limnephilidae	Trichoptera	Popintsi
Hirudinea			Annelida	Popintsi; Katunitsa
***Hydropsyche instabilis* (Curtis, 1834)**	*Hydropsyche* Pictet, 1834	Hydropsychidae	Trichoptera	Popintsi
***Hydroporus* sp.**	*Hydroporus* Clairville, 1806	Dytiscidae	Coleoptera	Popintsi
***Leuctra nigra* (Olivier, 1811)**	*Leuctra* Stephens, 1836	Leuctridae	Plecoptera	Popintsi
***Limnephilus* sp.**	*Limnephilus* Leach, 1815	Limnephilidae	Trichoptera	Popintsi
***Nepa cinerea*, larva Linnaeus, 1758**	*Nepa* Linnaeus, 1758	Nepidae	Hemiptera	Popintsi; Katunitsa
***Neureclipsis bimaculata* (Linnaeus, 1758)**	*Neureclipsis* McLachlan, 1864	Polycentropodidae	Trichoptera	Popintsi; Katunitsa
***Notonecta* sp.**	*Notonecta* Linnaeus, 1758	Notonectidae	Hemiptera	Popintsi

TAXON	GENUS	FAMILY	ORDER	BIOTOPE
***Physa acuta* Draparnaud, 1805**	*Physella* Haldeman, 1842	Physidae	Gastropoda	Katunitsa
TAXON	**GENUS**	**FAMILY**	**ORDER**	**BIOTOPE**
***Plectrocnemia conspersa* (Curtis, 1834)**	*Plectrocnemia* Stephens, 1836	Polycentropodidae	Trichoptera	Katunitsa
***Simulium* sp.**	*Simulium* Latreille, 1802	Simuliidae	Diptera	Popintsi; Katunitsa
***Stylodrilus heringianus* Claparède, 1862**	*Stylodrilus* Claparède, 1862	Lumbriculidae	Lumbriculida	Popintsi; Katunitsa
***Tabanus* sp.**	*Tabanus* Linnaeus, 1758	Tabanidae	Diptera	Popintsi
***Taeniopteryx nebulosa* (Linnaeus, 1758)**	*Taeniopteryx* Pictet, 1841	Taeniopterygidae	Plecoptera	Katunitsa
***Tipula* sp.**	*Tipula* Linnaeus, 1758	Tipulidae	Diptera	Popintsi; Katunitsa

In Popintsi biotope and Katunitsa biotope, respectively, 9 EPT taxa (39.13% of the established 23 macroinvertebrate taxa) and 10 EPT taxa (50% of the established 20 taxa) were found. Three Ephemeroptera taxa (with 261 specimens), one Plecoptera taxon (with 1 specimen) and five Trichoptera taxa (with 148 specimens) were found in Popintsi biotope. Five Ephemeroptera taxa (with 476 specimens), one Plecoptera taxon (with one specimen), and four Trichoptera taxa (with 148 specimens) were found in the Katunitsa biotope (Figure 2). Regarding the "number of EPT taxa", the ecological status of both rivers was "good".

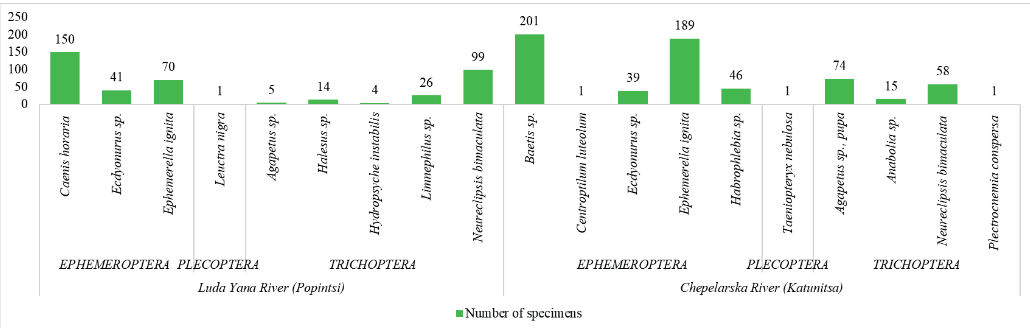

Figure 2. Number of EPT taxa and specimens from Popintsi biotope (Luda Yana River) and Katunitsa biotope (Chepelarska River)

Abundance of the benthic macroinvertebrate fauna

Oligochaeta taxa have not been identified in the Popintsi biotope. Three Diptera taxa (*Simulium* sp., *Tabanus* sp., and *Tipula* sp.; 7 specimens) were found. In the Katunitsa biotope, one Oligochaeta taxon (*Stylodrilus heringianus*; 14 specimens) and two Diptera taxa (*Simulium* sp. and *Tipula* sp.; 12 specimens) were established. Therefore, the % (Oligochaeta & Diptera) in the Popintsi biotope was 1.37% of the total abundance, and in the Katunitsa biotope - 3.65%.

In the Popintsi biotope, one taxon (*Simulium* sp.; 1 specimen) from the ecological group "filtering feeders" was found, representing 0.2% of the total abundance. In the Katunitsa biotope, one taxon (*Simulium* sp.; 9 specimens) was also found, i.e., the share of "filtering feeders" is 1.26% of the total abundance.

In Popintsi biotope, 9 Ephemeroptera, Plecoptera, and Trichoptera taxa were found, represented by 410 specimens. Meanwhile, in the Katunitsa biotope, 10 EPT taxa were established, represented by 625 specimens. Therefore, the % EPT taxa in the Popintsi biotope was 80.08% of the total abundance, and in the Katunitsa biotope – 87.78%.

To calculate the RETI trophic index, the trophic groups (SH - shredders; SC - scrapers;

FL - filtering feeders; CL - collectors; DF - deposit feeders) of the discovered taxa of benthic macroinvertebrate organisms (including the number of specimens) from the two studied biotopes were defined and presented. In Popintsi biotope, among the trophic groups of benthic macroinvertebrate organisms, the group of deposit feeders (DF; 159 specimens) was represented with the largest number of specimens, followed by the group of shredders (SH; 117 specimens), the group of scrapers (SC; 82 specimens), the group of collectors (CL; 4 specimens) and the group of filtering feeders (FL; 1 specimen). In the Katunitsa biotope, the group with the largest number of specimens was that of scrapers (SC; 352 specimens), followed by the group of shredders (SH; 256 specimens), the group of deposit feeders (DF; 14 specimens) and the group of filtering feeders (FL; 9 specimens). Taxa from the group of collectors (CL) were not found (Figures 3-4). According to the obtained value of the RETI index for the spring of 2023, the ecological status of the Luda Yana River (Popintsi biotope) was “good” (RETI = 0.548). The Chepelarska River (Katunitsa biotope) was in high ecological condition (RETI = 0.964).

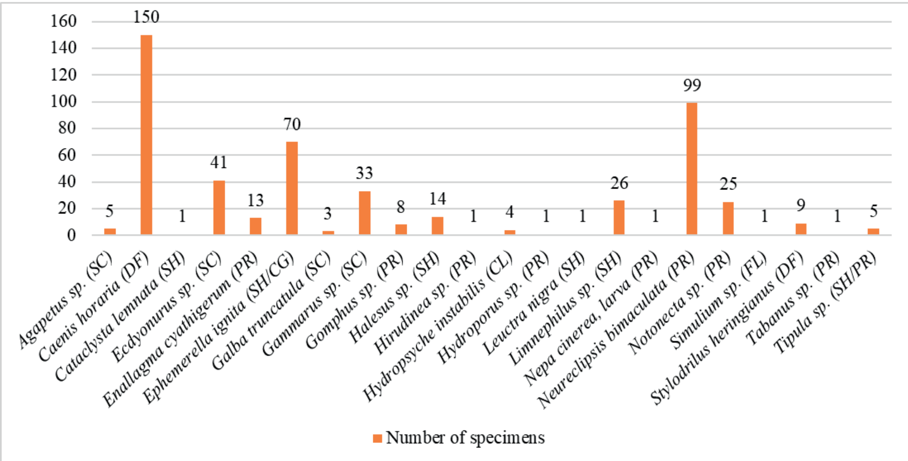

Figure 3. Trophic groups and number of specimens of macroinvertebrate taxa from Popintsi biotope (Luda Yana River)

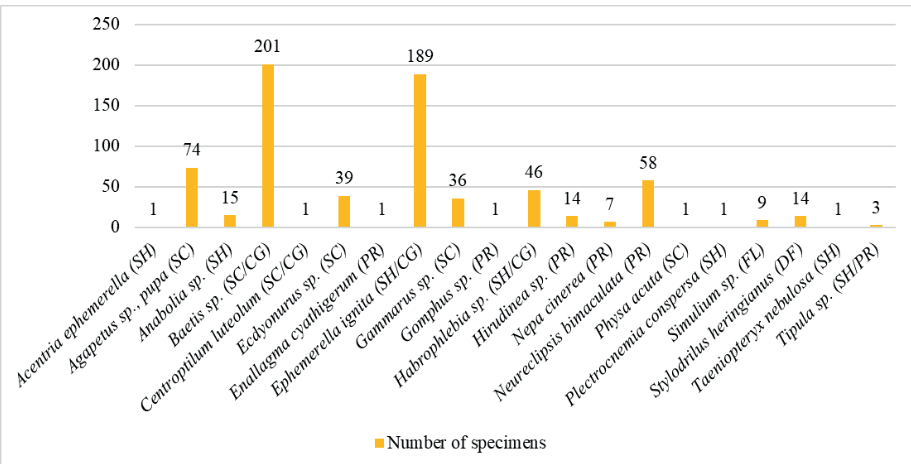

Figure 4. Trophic groups and number of specimens of macroinvertebrate taxa from Katunitsa biotope (Chepelarska River)

Saprobity

The detected 22 taxa (without Nematoda) and 20 taxa from Popintsi biotope and Katunitsa biotope, respectively, were found to belong to 8 saprobic groups: xeno-oligosaprobic group (χ-o); xeno-β-mesosaprobic group (χ-β); oligosaprobic group (o); oligo-β-mesosaprobic group (o-β); oligo-α- mesosaprobic group (o-α); oligo-polysaprobic group (o-p); β-mesosaprobic group (β); β-α- mesosaprobic group (β-α). In both biotopes, taxa belonging to the following saprobic groups were not found: xenosaprobic group (χ); α-mesosaprobic group (α), and polysaprobic group (p). The highest number of taxa and the highest number of specimens, both for Popintsi biotope and Katunitsa biotope, were found for the o-β saprobic group (Figure 5).

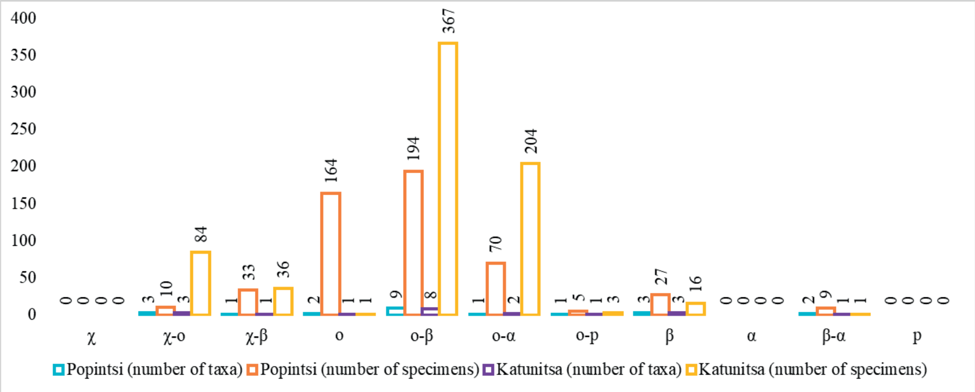

Figure 5. Distribution of detected taxa from Popintsi biotope and Katunitsa biotope by saprobic groups

Indices for species diversity

When the species richness index of Margalef has a value above 8, the ecosystem develops optimally (Kirin, 2015), and in the present study, the values of the index are smaller. In both biotopes, the Shannon-Weaver and Pielou index values correspond to β-mesosaprobia. Simpson's index values for both rivers are closer to 0, indicating more favorable conditions (Kirin, 2015) (Table 2).

Table 2. Indices for species diversity

Biotopes	Species richness index of Margalef (Dmg)	Shannon-Weaver species diversity index (H')	Pielou's evenness index (E)	Simpson's dominance index (C)
Popintsi (Luda Yana River)	3.37	2.2	0.711	0.16
Katunitsa (Chepelarska River)	2.89	2.06	0.688	0.179

Integrated indices for the benthic macroinvertebrate fauna (Adapted Biotic Index)

An Adapted Biotic index (BI) was calculated, for which the detected macroinvertebrate taxa were divided into sensitivity groups: Group A (sensitive), Group B (less sensitive), Group C (relatively tolerant), Group D (tolerant), and Group E (most tolerant). In both biotopes, the detected macroinvertebrate taxa referred to four groups of sensitivity – groups A, B, C, and D. The indicator Group C was represented with the largest number of taxa and the largest number of specimens (Figure 6). In conclusion, the Biotic Index for the Luda Yana River (Popintsi) had a value of 3, corresponding to a moderate ecological condition. At the same time, for the Chepelarska River (Katunitsa), the Biotic Index was equal to 4, i.e., the ecological status is very good.

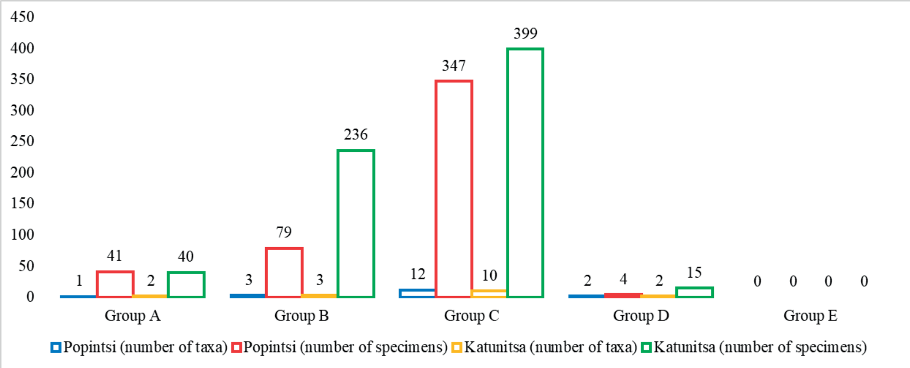

Figure 6. Distribution of detected taxa from Popintsi biotope and Katunitsa biotope by sensitivity groups

Discussions

Based on the published data from the East Aegean River Basin Directorate on the surface water state in the period 2014-2021, it was established that in the section of the Luda Yana River from the town of Panagyurishte to the confluence of the Strelchanska Luda Yana River (where Popintsi biotope is located), the ecological status of the water was "bad" and the chemical status - "good". As a result of the report on the water state of the Chepelarska River in the section of Assenovgrad town to the mouth and the Krumovsky collector (where Katunitsa biotope is located) for the period 2014-2020, a "bad" ecological and "bad" chemical status was established. Excesses of Cd and Pb were reported; as well as single excesses of Zn, Mn, and Ni (East Aegean River Basin Directorate, 2018). The current study of the ecological status of the water of the Luda Yana River (Popintsi) and the Chepelarska River (Katunitsa) based on BQE macrozoobenthos shows an improvement of the ecological status of the water of the two rivers in the studied sections in the spring of 2023. It suggests that the better ecological status of the water of the two river ecosystems is due to the higher water level, which is characteristic of the period during which the research was carried out.

CONCLUSIONS

The hydrobiological monitoring of the freshwater ecosystems of the Luda Yana River (Popintsi biotope) and the Chepelarska River (Katunitsa biotope) was carried out based on the biological quality element macrozoobenthos. According to the applied methodology and the calculated indices, it can be concluded that the ecological status of the Luda Yana River (Popintsi) was moderate ecological condition. In contrast, the ecological status of the Chepelarska River (Katunitsa) was good during the spring when the research was carried out.

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HELMINTOLOGICAL STUDY OF FISH FROM THE FRESHWATER ECOSYSTEMS OF THE LUDA YANA RIVER AND CHEPELARSKA RIVER

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Abstract

In the autumn of 2022, an ecologohelinological study was carried out on 41 and 70 fish specimens, respectively, from the Luda Yana River and the Chepelarska River, falling into the Maritsa River basin in the territory of Bulgaria. The fish from the Luda Yana River were caught from three places (indicated as biotopes) - Popinitsi, Svoboda, and Chernogorovo, and belong to 3 species (Orpheus dace, Squalius orpheus Kottelat & Economidis, 2006; Round-scaled barbel, Barbus cyclolepis Heckel, 1837 and Bleak, Alburnus alburnus Linnaeus, 1758). The fish from the Chepelarska River were caught from one place - the Katunitsa biotope and belong to 4 species (Sq. orpheus, B. cyclolepis, Alb. alburnus, Vardar nase Chondrostoma vardarense Karaman, 1928). In both studied rivers, the dominant fish species caught is Sq. orpheus. The species diversity of the parasites and their ecological indicators are indicated for the non-dominant fish species. New habitats and new hosts have been discovered for some of the established helminth species.

***Key words**: Bulgaria, Cyprinidae, ecological indices, helminths, Maritsa River Basin.*

INTRODUCTION

The Luda Yana River and the Chepelarska River are among the major tributaries of the Maritsa River on Bulgarian territory. The Luda Yana River (with a length of 74 km) rises from Sredna Gora Mountain, enters the Upper Thracian Plain, and flows from the left into the Maritsa River, north of the village of Sinitovo. The Chepelarska River (with a length of 86 km) rises from the Rhodope Mountain, enters the Upper Thracian Plain (in the region of the town of Assenovgrad), and flows from the right into the Maritsa River between the town of Plovdiv and the town of Sadovo (East Aegean River Basin Directorate, 2018). Three (Orpheus dace, *Squalius orpheus* Kottelat & Economidis, 2006; round-scaled barbell, *Barbus cyclolepis* Heckel, 1837 and bleak, *Alburnus alburnus* Linnaeus, 1758) of the four fish species subject to helminthological research in the present study are included in the IUCN Red List with the "LC" category. One species (*Chondrostoma vardarense* Karaman, 1928) is in the "NT" category. *B. cyclolepis* is protected by the Biological Diversity Act and the Habitats Directive. *Sq. orpheus*, *B. cyclolepis* and *Ch. vardarense* are endemic to Europe (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; IUCN, 2024). Scientific studies on the species composition, the parasite ecological indices, and the parasite communities of fish from the Luda Yana River are scarce (Kirin, 2002b; Kirin et al., 2019; Zaharieva, 2023a). At present, the parasite fauna of fish from the Chepelarska River has been studied by Kakacheva-Avramova (1965), Kirin (2002a), Chunchukova (2020), and Zaharieva (2023b).

The purpose of the present study is to update the existing and provide new data on the helminth species diversity and the structure of helminth communities of fish from the freshwater ecosystems of the Luda Yana River and Chepelarska River, flowing into the Maritsa River on the territory of Bulgaria.

MATERIALS AND METHODS

A total of 111 specimens of fish from 4 species (Orpheus dace, *Squalius orpheus* Kottelat & Economidis, 2006; round-scaled barbell, *Barbus cyclolepis* Heckel, 1837; bleak, *Alburnus alburnus* Linnaeus, 1758; *Chondrostoma vardarense* Karaman, 1928), caught from two rivers flowing into the basin of the Maritsa River, were subjected to helminthological examination (Table 1).

Table 1. Caught fish specimens from the Luda Yana River and the Chepelarska River

Fish species	Luda Yana River N = 41	Chepelarska River N = 70
Squalius orpheus Kottelat & Economidis, 2006	N = 34	N = 51
Barbus cyclolepis Heckel, 1837	N = 5	N = 16
Alburnus alburnus (Linnaeus, 1758)	N = 2	N = 2
Chondrostoma vardarense Karaman, 1928	-	N = 1

For the research, three places (designated as biotopes) along the course of the Luda Yana River were visited - in the vicinity of the village of Popintsi, the village of Svoboda, and the village of Chernogorovo. Popintsi biotope is located along the course of the Luda Yana River before it enters the village of Popintsi (42°25'26.8"N 24°16'02.5"E; Panagyurishte Municipality; Pazardzhik Province). Svoboda biotope is located next to the bridge over the Luda Yana River on the road between the villages Svoboda and Popintsi, shortly before the Radka mine (42°24'07.3"N 24°17'24.2"E; Strelcha Municipality; Pazardzhik Province). Chernogorovo biotope is located next to the bridge over the Luda Yana River, southwest of the village of Chernogorovo on the road in the direction of the town of Pazardzhik (42°16'12.9"N 24°23'33.3"E; Pazardzhik Municipality; Pazardzhik Province) (Figure 1).

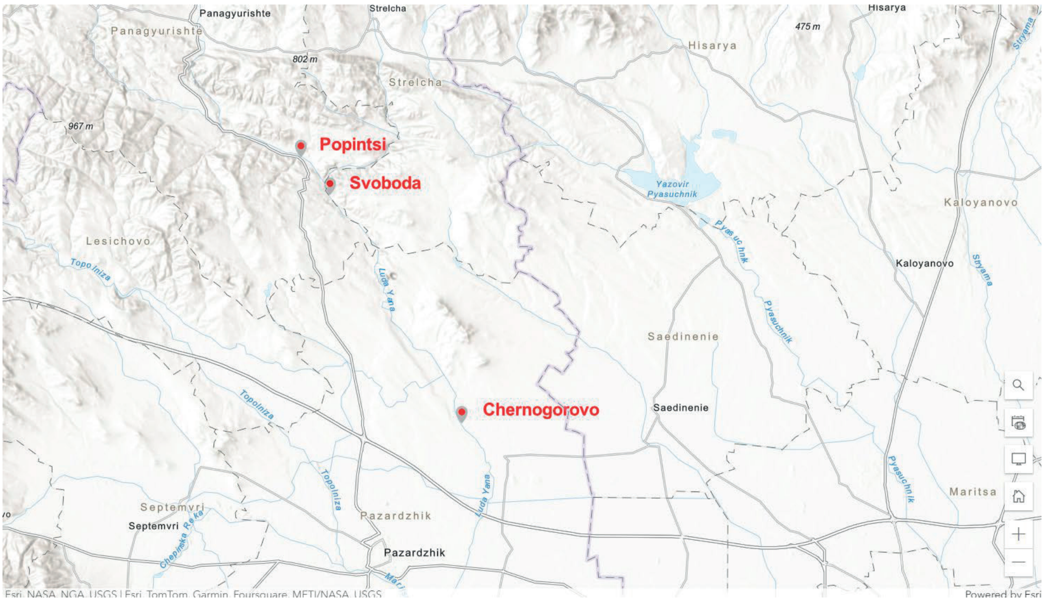

Figure 1. Researched biotopes from the Luda Yana River (https://www.esri.com/en-us/arcgis/products/arcgis-online/overview)

In connection with the study, two places were visited along the Chepelarska River - in the vicinity of the villages of Bachkovo and Katunitsa. Katunitsa biotope is located along the Chepelarska River in the western outskirts of the village of Katunitsa between the railway bridge and the concrete bridge (42°06'08.3"N 24°51'58.8"E; Sadovo Municipality; Plovdiv

Province). Bachkovo biotope includes two places located along the river between the village of Bachkovo and the town of Assenovgrad (41°57'52.5"N 24°51'57.0"E and 41°57'38.0"N 24°51'47.5"E; Assenovgrad Municipality; Plovdiv Province). Fish populations were not detected from the Bachkovo biotope during the study period (Figure 2).

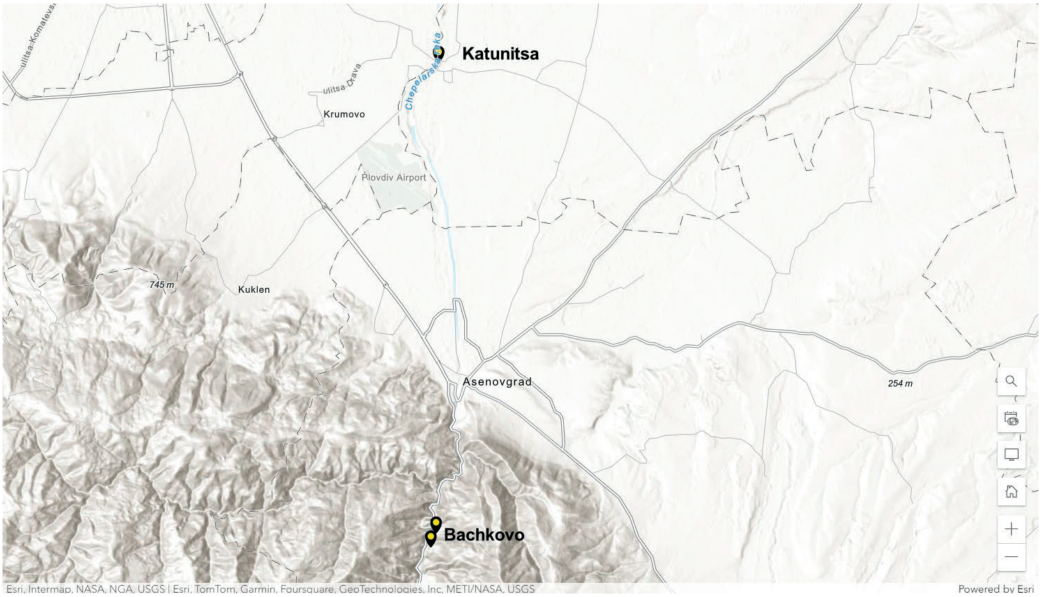

Figure 2. Researched biotopes from the Chepelarska River
(https://www.esri.com/en-us/arcgis/products/arcgis-online/overview)

The fish were caught according to the requirements of the Ministry of Agriculture and the Executive Agency for Fisheries and Aquaculture (EN 14757:2015). The catching of the fish was carried out after issuing a license to catch fish for scientific research purposes and a ticket for amateur fishing. The fish species was determined in the field (according to Karapetkova & Zhivkov, 2006; Froese & Pauly, 2023) and basic metric data (total body length - TL in centimeters; maximum body height - MH in centimeters; body weight - BW in grams) were recorded. The largest number of fish specimens were caught from the Katunitsa and Popintsi biotopes (Table 2).

The fish subjected to helminthological examination were collected in the autumn of 2022.

Table 2. Metric data of caught fish specimens by biotopes

River	Biotope	Fish species	TL (mean ± SD)	MH (mean ± SD)	BW (mean ± SD)
Luda Yana	Popintsi	*Squalius orpheus* (N = 32)	13.16 ± 3.22	2.71 ± 0.76	20.29 ± 12.38
	Svoboda	*Squalius orpheus* (N = 1)	6.3	1.3	2
		Barbus cyclolepis (N = 5)	9.1 ± 0.6	1.82 ± 0.23	5.6 ± 2.07
		Alburnus alburnus (N = 2)	10.2 ± 0.14	2.05 ± 0.07	6 ± 0.00
	Chernogorovo	*Squalius orpheus* (N = 1)	9.2	1.7	6
Chepelarska	Katunitsa	*Squalius orpheus* (N = 51)	13.74 ± 3.29	2.86 ± 0.74	24.64 ± 14.92
		Barbus cyclolepis (N = 16)	9.13 ± 4.64	2.23 ± 0.47	11.93 ± 8.05
		Alburnus alburnus (N = 2)	12.6 ± 0.28	2.6 ± 0.42	14 ± 2.83
		Chondrostoma vardarense (N = 1)	17	3.6	42
	Bachkovo	-	-	-	-

Immediately after catching 111 fish specimens, the body surface, the abdominal cavity, and the internal organs were examined for parasites. The collected host material samples were preserved in 70% ethyl alcohol and prepared for transport and subsequent laboratory processing. The helminthological examination was carried out according to standard methods (Zashev & Margaritov, 1966; Moravec, 2013; and others). For species identification of helminths, temporary microscopic preparations of the representatives of classes Acanthocephala and Nematoda (Zashev & Margaritov, 1966; Moravec, 2013) and permanent microscopic preparations of classes Trematoda and Cestoda (Georgiev et al., 1986; Scholz & Hanzelova, 1998) were prepared. The species of the isolated helminths were determined according to Bauer (Ed.) (1987), Moravec (2013), and others. Basic ecological indices were calculated, such as the number of infected fish and fish parasites, mean intensity, mean abundance, and prevalence (Bush et. al, 1997).

RESULTS AND DISCUSSIONS

Structure of the helminth communities

During the examination of a total of 111 fish specimens from the Luda Yana River and Chepelarska River, 12 helminth taxa (*Nicolla skrjabini* (Iwanitzky, 1928) Dollfus, 1960; *Caryophyllaeus brachycollis* Janiszewska, 1953; *Caryophyllaeides fennica* (Schneider, 1902) Nybelin, 1922; *Acanthocephalus anguillae* (Müller, 1780) Lühe, 1911; *Acanthocephalus lucii* (Müller, 1776) Lühe, 1911; *Acanthocephalus tenuirostris* (Achmerov & Dombrovskaja-Achmerova, 1941) Yamaguti, 1963); *Pomphorhynchus laevis* (Zoega in Müller, 1776) Porta, 1908; *Contracaecum* sp.; *Schulmanela petruschewskii* (Schulman, 1948) Ivashkin, 1964; *Philometra ovata* (Zeder, 1803); *Raphidascaris acus* (Boch, 1779); *Rhabdochona denudata* (Dujardin, 1845) Railliet, 1916), belonging to 4 classes (Trematoda, Cestoda, Acanthocephala, Nematoda) were found (Table 3).

Table 3. Taxonomic position, fish host, and biotope of the established helminth specimens

Helminth	Taxonomic position	Fish host	Biotope
Nicolla skrjabini (Iwanitzky, 1928) Dollfus, 1960	CLASS TREMATODA RUDOLPHI, 1808 Family Opecoelidae Ozaki, 1925 Genus *Nicolla* Wišniewski, 1933	*Barbus cyclolepis*	Svoboda
Caryophyllaeus brachycollis Janiszewska, 1953	CLASS CESTODA RUDOLPHI, 1808 Family Caryophyllaeidae Leuckart, 1878 Genus *Caryophyllaeus* Müller, 1878	*Barbus cyclolepis*	Katunitsa
Caryophyllaeides fennica (Schneider, 1902) Nybelin, 1922	CLASS CESTODA RUDOLPHI, 1808 Family Lytocestidae Wardle and McLeod, 1952 Genus *Caryophyllaeides* Nybelin, 1922	*Squalius orpheus*	Popintsi
		Barbus cyclolepis	Svoboda
Acanthocephalus anguillae (Müller, 1780) Lühe, 1911	CLASS ACANTHOCEPHALA (RUDOLPHI, 1808) Family Echinorhynchidae (Cobbold, 1879) Hamann, 1892 Genus *Acanthocephalus* Koelreuther, 1711	*Squalius orpheus*, *Barbus cyclolepis*	Katunitsa
Acanthocephalus lucii (Müller, 1776) Lühe, 1911		*Squalius orpheus*	Popintsi
Acanthocephalus tenuirostris (Achmerov & Dombrovskaja-Achmerova, 1941) Yamaguti, 1963)		*Squalius orpheus*, *Barbus cyclolepis*	Katunitsa
Pomphorhynchus laevis (Zoega in Müller, 1776) Porta, 1908	CLASS ACANTHOCEPHALA (RUDOLPHI, 1808) Family Pomphorhynchidae Yamagiti, 1939 Genus *Pomphorhynchus* Monticelli, 1905	*Squalius orpheus*	Chernogorovo
Contracaecum sp.	CLASS NEMATODA RUDOLPHI, 1808 Family Anisakidae Skrjabin et Karokhin, 1945 Genus *Contracaecum* Railliet & Henry, 1912	*Squalius orpheus*	Katunitsa

Helminth	Taxonomic position	Fish host	Biotope
Schulmanela petruschewskii (Schulman, 1948) Ivashkin, 1964	CLASS NEMATODA RUDOLPHI, 1808 Family Capillariidae Railliet, 1915 Genus *Schulmanela* Ivashkin, 1964	*Barbus cyclolepis*	Katunitsa
Philometra ovata (Zeder, 1803)	CLASS NEMATODA RUDOLPHI, 1808 Family Philometridae Baylis et Daubney, 1926 Genus *Philometra* Costa, 1845	*Barbus cyclolepis*	Katunitsa
Raphidascaris acus (Boch, 1779)	CLASS NEMATODA RUDOLPHI, 1808 Family Raphidascarididae Genus *Raphidascaris* (Bloch, 1779) Railliet et Henry, 1915	*Alburnus alburnus*	Svoboda
		Squalius orpheus, *Barbus cyclolepis*	Katunitsa
Rhabdochona denudata (Dujardin, 1845) Railliet, 1916	CLASS NEMATODA RUDOLPHI, 1808 Family Rhabdochonidae Travassos, Artigas et Pereira, 1928 Genus *Rhabdochona* Railliet, 1916	*Squalius orpheus*	Popintsi
		Squalius orpheus	Katunitsa

Helminth communities of Squalius orpheus from the Luda Yana River and the Chepelarska River

During the examination of 32, 1, and 1 *Sq. orpheus* specimen from Popintsi biotope, Chernogorovo biotope, and Svoboda biotope, located along the Luda Yana River, a total of 4 helminth taxa were found - *C. fennica*, *Ac. lucii*, *Rh. denudata* and *P. laevis*. The studied *Sq. orpheus* specimen from Svoboda biotope was not infected. In the component community of Orpheus dace from Popintsi biotope, the representatives of class Acanthocephala (1 species with 15 specimens) had the largest number of specimens. They were followed by the representatives of class Nematoda (1 species with three specimens) and Cestoda (1 species with one specimen). One component helminth species (*Ac. lucii*) and two accidental helminth species (*C. fennica* and *Rh. denudata*) were found in the helminth community of *Sq. orpheus* from Popintsi biotope. For Popintsi biotope, *Ac. lucii* had the highest value for mean intensity (MI) and mean abundance (MA) (Figure 3).

During the ecologohelminthological examination of 51 *Sq. orpheus* specimens from Katunitsa biotope, located along the Chepelarska River, an infection with a total of 5 endohelminth species was found - *Ac. anguillae*, *Ac. tenuirostris*, *Contracaecum* sp., *R. acus*, 1. and *Rh. denudata*. In the component community of *Sq. orpheus* from Katunitsa biotope, the largest number of specimens had the nematodes (3 species with 38 specimens), followed by the acanthocephalans (2 species with 4 specimens). In the component community of *Sq. orpheus*, one core species (*Contracaecum* sp.) was found, while the remaining helminth species were accidental. *R. acus* had the highest value for MI. *Contracaecum* sp. had the highest value for MA (Figure 3).

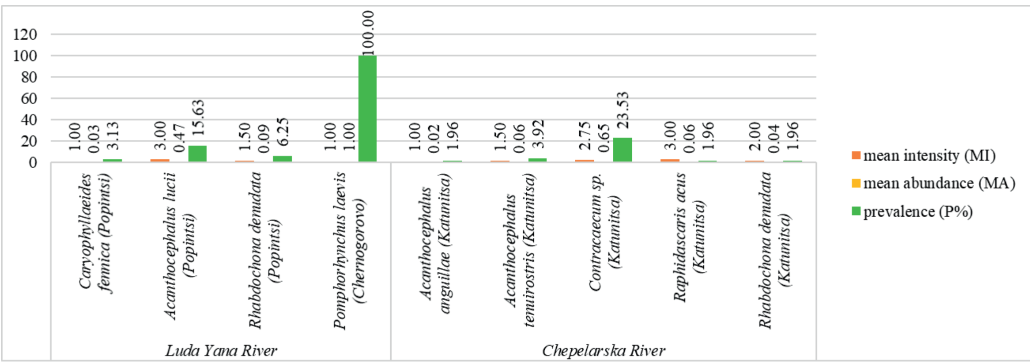

Figure 3. Ecological indices (MI, MA, P %) of *Sq. orpheus* helminths from the Luda Yana River and the Chepelarska River

There are few ecologohelminthological studies of *Sq. orpheus* from the Luda Yana River and the Chepelarska River.

The present article builds on and updates the existing data on the helminth fauna of *Sq. orpheus* from the two rivers, part of the Maritsa River Basin in Bulgaria (Zaharieva, 2023a, 2023b). Kirin et al. (2019) studied *Rutilus rutilus* Linaeus, 1758 from the Luda Yana River (Popintsi biotope) and reported the same three endohelminths species (*C. fennica*; *Ac. lucii* and *Rh. denudata*).

Chernogorovo biotope is a new habitat for *P. laevis* from *Sq. orpheus*. Kirin (2002a) reported *B. rectangulum*, *Ac. anguillae*, *Ac. tenuirostris*, *C. microcephalum* (l.) and *Rh. denudata* for *Sq. orpheus* from the Chepelarska River (between Assenovgrad and Bachkovo). Katunitsa biotope is a new habitat for *Ac. anguillae* and *R. acus* (l.) from Orpheus dace. *R. acus* (l.) is reported for the first time from *Sq. orpheus* from the Chepelarska River.

Helminth communities of Barbus cyclolepis from the Luda Yana River and the Chepelarska River

As a result of the ecologoparasitological examination of 5 *B. cyclolepis* specimens from Svoboda biotope, located along the Luda Yana River, two helminth species were found - one specimen each of *N. skrjabini* and *C. fennica*. A mixed infection was not found. In the component community of round-scaled barbell from the Svoboda biotope, the two established helminth species had equal values of the ecological indices (Figure 4).

In the current helminthological study of 16 *B. cyclolepis* specimens from Katunitsa biotope from the Chepelarska River, infection with six endohelminth species was found - *C. brachycollis*, *Ac. anguillae*, *Ac. tenuirostris*, *Ph. ovata*, *R. acus* (adult and l.), *Sch. petruschewskii*. In round-scaled barbell from Katunitsa biotope, *R. acus* had the highest ecological indices (Figure 4).

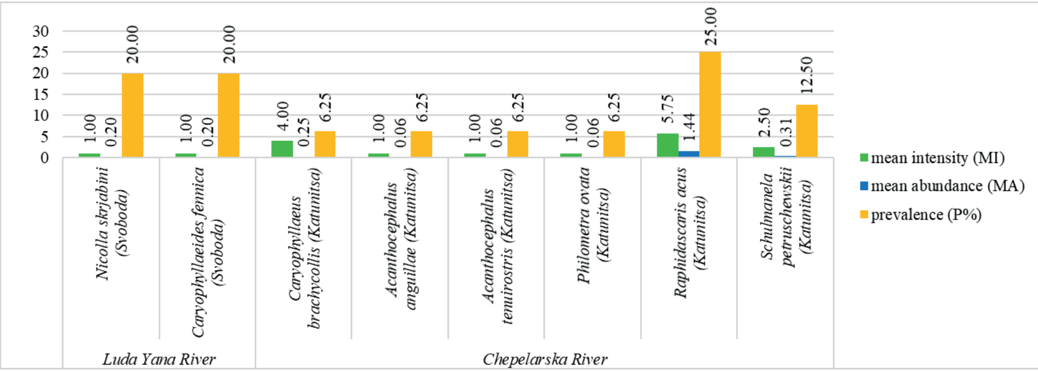

Figure 4. Ecological indices (MI, MA, P %) of *B. cyclolepis* helminths from the Luda Yana River and the Chepelarska River

Kirin (2002b) investigated *B. cyclolepis* (syn. *Barbus tauricus cyclolepis*) from the Luda Yana River (Panagyurishte biotope) and reported four helminth species - *All. isoporum*, *C. brachycollis*, *B. rectangulum*, *Ac. anguillae*. Svoboda biotope (Luda Yana River) is a new habitat for the two helminth species (*N. skrjabini* and *C. fennica*) from *B. cyclolepis* that are established in the present study. Katunitsa biotope is a new habitat for the six endohelminth species found in *B. cyclolepis*. *N. skrjabini*, *Ac. tenuirostris*, *Ph. ovata* and *R. acus* are reported for the first time from *B. cyclolepis* and the Aegean Water Basin in Bulgaria.

Helminth communities of Alburnus alburnus from the Luda Yana River and the Chepelarska River, and Chondrostoma vardarense from the Chepelarska River

During the helminthological examination of two *Alb. alburnus* specimens from the Svoboda biotope (Luda Yana River) found that only one specimen was infected. One helminth species was found (*R. acus*) with ecological indices: MI = 1.00; MA = 0.50; P% = 50.00.

Until now, there have been no ecoparasitological studies on *Alb. alburnus* from the Luda Yana River. Svoboda biotope is a new habitat for *R. acus* from bleak. *R. acus* is reported for the first time from *Alb. alburnus* from the Aegean Water Basin in Bulgaria. The examined two *Alb. alburnus* specimens and one *Ch. vardarense* specimen from Katunitsa biotope (Chepelarska River) were not infected.

CONCLUSIONS

For the current helminthological study, three biotopes were visited, located along the Luda Yana River, and three fish species (*Sq. orpheus*, *B. cyclolepis*, *Alb. alburnus*) were caught. Infection with a total of 6 endohelminth species (*N. skrjabini*, *C. fennica*, *A. lucii*, *P. laevis*, *R. acus*, *Rh. denudata*) was found. In addition, two biotopes were visited, located in the lower reaches of the Chepelarska River, and fish were caught only from Katunitsa biotope. Four fish species (*Sq. orpheus*, *B. cyclolepis*, *Alb. alburnus*, *Ch. vardarense*) were collected and infection with a total of 8 endohelminth species (*Ac. anguillae*, *Ac. tenuirostris*, *C. brachycollis*, *Contracaecum* sp., *Ph. ovata*, *R. acus*, *Rh. denudata*, *Sch. petruschewskii*) was found. In the helminthological studies on fish from both rivers, the highest species diversity of helminths was found in *Sq. orpheus*.

As a result of the research, the following conclusions can be made:

- ✓ Chernogorovo biotope (Luda Yana River) is a new habitat for *P. laevis* from *Sq. orpheus*.
- ✓ Svoboda biotope (Luda Yana River) is a new habitat for *N. skrjabini* and *C. fennica* from *B. cyclolepis*.
- ✓ Svoboda biotope (Luda Yana River) is a new habitat for *R. acus* from *Alb. alburnus*.
- ✓ Katunitsa biotope (Chepelarska River) is a new habitat for *Ac. anguillae* and *R. acus* (l.) from *Sq. orpheus*.
- ✓ Katunitsa biotope (Chepelarska River) is a new habitat for *C. brachycollis*, *Ac. anguillae*, *Ac. tenuirostris*, *Ph. ovata*, *R. acus* (adult and l.) and *Sch. petruschewskii* from *B. cyclolepis*.
- ✓ N. *skrjabini*, *Ac. tenuirostris, Ph. ovata*, and *R. acus* are reported for the first time for *B. cyclolepis* from the Aegean Water Basin in Bulgaria
- ✓ *R. acus* is reported for the first time for *Alb. alburnus* from the Aegean Water Basin in Bulgaria.
- ✓ *R. acus* (l.) is reported for the first time for *Sq. orpheus* from Chepelarska River.

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