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

SCIENTIFIC PAPERS
SERIES D. ANIMAL SCIENCE

Volume LXIV, No. 1

2021
BUCHAREST

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PUBLISHERS:
University of Agronomic Sciences and Veterinary Medicine of Bucharest, Romania – Faculty of Animal Science Engineering and Management
Address: 59 Mărăști Blvd, District 1, 011464, Bucharest, Romania
Phone: + 40 213 182 564, Fax: +40 213 182 888**,** www.zootehnie.ro

CERES Publishing House
Address: 29 Oastei Street, District l, Bucharest, Romania
Phone: + 40 317 90 23, E-mail: edituraceres@yahoo.com, Webpage: www.editura-ceres.ro

To be cited: Scientific Papers. Series D. Animal Science, Volume LXIV, No. 1, 2021

ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

International Database Indexing: Web of Science Core Collection (Emerging Sources Citation Index), Index Copernicus, CABI, DOAJ, Ulrich's Periodicals Directory (ProQuest), PBN, Cite Factor (Academic Scientific Journals), Scipio, OCLC (WorldCat), Research Bible, Google Scholar.

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GENETICS AND BREEDING

Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

EVALUATION OF THE EFFECT OF SOME ABIOTIC FACTORS ON THE WEIGHT DEVELOPMENT OF YOUNG FEMALE ANIMALS OF THE ILE DE FRANCE BREED IN BULGARIA

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Abstract

Subject of the study were 655 female lambs from the Ile de France breed, born in the period 2017-2020 in Bulgaria. Weight development was monitored. Live weight at birth, at 30, at 70 days and 9 months was measured. The gain for the studied periods was calculated. The analysis was made using a multifactor linear-statistical model for each studied age. The factors inducing a specific variance were year of birth, lambing season, and farm. The year had a significant effect on the live weight at all studied ages, except weight at birth. The season affected live weight at all levels, excluding weight at 30 days. The average daily gain indicated a reliable specific variance depending on the year of birth of all studied ages. The season affected the gain of 30 to 70 days only. The farm had a highly significant effect on the live weight and the gain at all ages. The obtained results for live weight and gain from birth to 9 months of age confirmed the good adaptation of the breed in Bulgaria and the opportunity for realization its potential for high growth intensity at an early age.

Key words*: average daily gain, farm, Ile de France sheep breed, live weight, season, year of birth.*

INTRODUCTION

Interest in the sheep breeding meat industry in our country has significantly increased in recent years. The reason is the growing relative share of revenue from the sale of breeding animals and lambs for meat, compared to other revenues. This trend is observed in all areas of sheep breeding. The creation of the meat-producing sheep breed Ile de France began in 1920, and the first import in Bulgaria was realized in 1968. The Association for Breeding Ile de France Sheep in Bulgaria (ABILFB) conducts breeding activities with 6,200 purebred animals under selection control and about 1,600 crosses in 2020. According to France Génétique Elevage (2019), about 230,000 Ile de France sheep are bred in France, and the breed is bred in over 50 countries on all continents (INSEM OVIN, 2020). The specialized meat breed is characterized by intensive growth at an early age. The meat has excellent taste, marbled, without the characteristic specific odour of some other breeds raised in our country. Good growth abilities, meat-producing qualities and feed consumption are in optimal combination at slaughter level from 35 kg to 40 kg, i.e. the breed specializes in the production of heavy-type lambs. The productivity and condition of the Ile de France sheep population in Bulgaria have been studied by Bulgarian authors (Achkakanova et al., 2019; 2020; Dimitrov, 1978; 1991; Dimitrov et al. 1987; 2011; Ivanova, 2020; Ivanova et al., 2017; Laleva, 1996; Laleva et al., 2020; Metodiev et al., 2008; 2010; Raycheva et al., 2005; 2010). Other authors explore the possibilities of crossing with our breeds and improving their meat-producing qualities, respectively and the economic effect of breeding (Dimitrov, 1988; Laleva et al., 2006; Marinova, 1976; Slavov, 2007). The tendency for expansion of the Ile de France population, as the main meat-producing breed in Bulgaria, motivates our research. Studies on the influence of environmental and genetic factors are needed, both in purebred herds and in crosses with improved meat production qualities.

The aim of the study was to establish the effect of some abiotic factors on weight development

of young female animals from the Ile de France breed in Bulgaria.

MATERIALS AND METHODS

Subject of the study were 655 female lambs from the Ile de France breed. The animals were born in the period 2017-2020 and were raised in three farms in North Bulgaria. Weight development from birth to 9 months of 655 female lambs was monitored. For this purpose, the following were measure: live weight at birth, at 30 days, at 70 days and at 9 months. Live weight was measured with precision up to 0.1 kg. Average daily gain of animals, realized in the studied periods, was calculated. The data were obtained from the Pedigree Books, which were updated by breeders from the Association for Breeding Ile de France Sheep in Bulgaria. Data were obtained using standard methods and instructions provided in the Instruction for control of productive traits and grading, which is part of the selection program for the development of the breed in our country. The analysis of the variance is made on the basis of a multifactor linear-statistical model for each studied age, which has the following form:

$$\mathbf{Y_{ijklm} = \mu + A_{ijkl} + B_{mn} + C_{opq} + e_{ijklmnopq}}$$

In which:

μ - total average for all ages

A_{ijkl} – Effect of the factor year of birth (fixed) - 4 levels (2017-2020)

$\mathbf{B_{mn}}$ - Effect of the factor season (fixed) - 2 levels (1 - spring; 2 - autumn)

$\mathbf{C_{opq}}$ - Effect of the factor farm (fixed) - 3 levels (1-3)

$\mathbf{e_{ijklmnopq}}$ - residual effects, $\approx N (O, \delta e^2)$

The differences between levels of the studied traits were established on the basis of the distribution values, calculated with the Studant test (Hayter A., 1984):

$$(y_i - y_j)/S \sqrt{(1/n_i + 1/n_j)/2}$$

In which:

$(y_i - y_j)$ - difference between average values for the levels of the studied trait

S - Standard deviation

n_i, n_j - Number of observation (animals) for corresponding levels

RESULTS AND DISCUSSIONS

The year of birth had a significant effect ($P<0.001$) on the live weight trait of Ile de France female lambs at all studied ages, except weight at birth (Table 1). This factor is a complex abiotic factor and source of specific variance on live weight. The year of birth indicates the influence of all components of the environment in the specific year that affect the weight development lambs after birth. The lambing season also affected all ages ($P<0.05$, $P<0.01$, $P<0.001$), excluding weight at 30 days. The farm had a highly significant effect ($P<0.001$) on live weight and the average daily gain at all ages of the studied selection animals. The values of the F criterion for the effect of the year vary from 21,350 to 50,496, for the factor season up to 48,431 and for the farm it reached 371,613 at 70 days. The coefficients of variation of the studied trait range from 22.12% at birth to 8.36%, gradually decreasing with age. The high level of variation in birth weight is logical in a breed with high fertility, due to the significant differences between offspring with different types of birth. The coefficients of determination of the used model varied from 0.420 to 0.849 at different ages, which indicates that a significant part of the variation is due to the sources of variability included in the model. Achkakanova et al. (2020) found a significant effect from the factor farm, combined with genetic factors, on the weight development of male and female animals up to 70 days. The authors believe that when calculating breeding values, the interaction between the individual factors should be taken into account and the farm, year and month of lambing should be included in the linear models. Dimitrov (1978a) did not establish significant differences in the weight development of lambs for breeding, obtained from imported sheep and purebred animals raised in our country. The author proved a significant influence of the factor year of birth to weaning, which was confirmed by our study (Dimitrov, 1978b). Dimitrov et al. (1982) showed a significant effect of the factor year on live weight at birth.

Table 2 shows a significant specific variance of the realized gain by periods depending on the year and the farm for the three studied periods

(P<0.001) and the season 30-70 days (P<0.001).

Table 1. Analysis of variance of the trait live weight of female lambs

Factors	df	F	P	R^2	CV%
1 day					
Year of birth	3	1,989	n.s.	0.420	22.12
Season	1	6,359	*		
Farm	2	31,041	***		
30 days					
Year of birth	3	34,253	***	0.579	12.19
Season	1	0.033	n.s.		
Farm	2	67,211	***		
70 days					
Year of birth	3	50,496	***	0.849	10.60
Season	1	48,431	***		
Farm	2	371,613	***		
9 months					
Year of birth	3	21,350	***	0.516	8.36
Season	1	7,617	**		
Farm	2	35,281	***		

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

Table 2. Analysis of variance of the trait average daily gain of female lambs

Factors	df	F	P	R^2	CV%
1 day - 30 days					
Year of birth	3	42,958	***	0.514	16.35
Season	1	1,609	n.s.		
Farm	2	46,000	***		
30 days - 70 days					
Year of birth	3	102,560	***	0.815	22.58
Season	1	52,342	***		
Farm	2	220,888	***		
70 days - 9 months					
Year of birth	3	14,913	***	0.508	16.89
Season	1	2,569	n.s.		
Farm	2	27,047	***		

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

The values of the F criterion for the effect of the year reached 102,560, and for the farm up to 220,888 for the second period of the studied gain. The variation of the studied trait is from 16.35% to 22.58%. The coefficients of determination of the used model ranged from 0.508 to 0.815 at different ages and this indicated a good representativeness of the results. Achkakanova and Staykova (2019b) found that the farm and the year of birth have a significant impact on the realized gain by periods, except for the year after 9 months.

The results in Table 3 show that animals born in 2019 have higher live weight values compared to other groups of all studied ages (P<0.05, P<0.01, P<0.001). It is noteworthy that the superiority increases with age. For those born in 2020, a positive LS-assessment was observed at birth (P<0.05, P<0.01, P<0.001), but at following ages the average weight progressively decreased compared to their peers. Those born in 2017 and 2018 showed negative LS- assessments at birth, but were 30 days ahead of their peers in live weight (P<0.05, P<0.01, P<0.001), which was probably due to a positive maternal effect. At following ages, they had different deviations from the average weight. Female lambs born in the spring definitely dominated in live weight at all studied ages, compared to the autumn offspring (P<0.05, P<0.001). The analysis of farm data indicated the strong influence of this factor at all ages. The results of the analysis of the variance, where the values of the F criterion are high, were confirmed. Female offspring from Farm 1 were significantly ahead of their peers at all ages (P<0.001). The lambs from Farm 2 showed a positive deviation at birth, but then lagged behind the average for the studied selection of animals, together with the animals from Farm 3. They were characterized by negative LS-assessments for live weight at all ages. The average live weight of female lambs at birth was 4,660 kg, at 30 days - 14,890 kg, at 70 days - 27,861 kg and at 9 months - 56,341 kg (Table 3).

Close to our results for the average live weight of female lambs, individuals of this breed at 30 days were established in France - 13,900 kg, and at 70 days - 27,200 kg. The information was published in the Yearbook of the National Institute of Animal Husbandry in France, which conducted its research in collaboration with INRAe - Bilan du Contrôle de Performances Ovins Allaitants - Campagne 2019, Institut de l'Elevage/IDELE/INRAe, Races de France. For

twins, the results were lower by 14.4% and 10.3% at the same ages, respectively. In total, for both sexes, the data for 2018 show an average weight of 14.500 kg at 30 days and 28,900 kg at 70 days (Résultats du contrôle de performances 2018, Institut de l'Elevage & Races de France). Raycheva et al. (2005) published average values for live weight - at birth (4,370 kg), which is close to our result. For following ages the values were lower - at 30 days (11,826 kg) and at 70 days (20,750 kg). Laleva et al. (2006) published data on an average weight at birth (3,570 kg), and at other ages they were close to those of Raycheva et al. (2005). Laleva et al. (2020) published a study with lower values for average live weight from birth to 70 days for the herd in Agricultural institute - Stara Zagora, while the values for animals from Institute of Animal Sciences - Kostinbrod were closer to our results at birth (5.005 kg), but for following ages were lower than our study. Achkakanova and Staykova (2019a) found an average live weight of 70,939 kg at 2 years of age, compared to female Ile de France lambs at 9 months in our study which reached 79.42% of the weight of adult animals. This is indicative of the high growth rate at a young age and precocity of the breed, which is a sufficient condition for early conception after this age. Achkakanova et al. (2020) reported close to our live weight data of female lambs of the same age up to 70 days. Our results are close to those in the report of the Association for Breeding of the Ile de France sheep in Bulgaria for 2019 for the entire population bred in our country. These results are similar to the realized live weight and gain of the young animals of the breed in France, which proves that the adaptation under our conditions is successful and the full productive potential of the Ile de France sheep can be realized.

Table 3. LS-estimates (LSC) of the: year of birth, season and farm effects on the live weight of female lambs at different age

Age	1 day			30 days			70 days			9 months		
	n	LSC	SE	n	LSC	SE	n	LSC	SE	n	LSC	SE
Year of birth												
2017	313	-0.016	0.066	313	1.182**ABC**	0.121	313	-1.079 **aA**	0.188	312	1.125 **aA**	0.313
2018	91	-0.251 **l**	0.106	91	0.228**AD**	0.194	91	0.345**aBb**	0.304	82	-0.139 **Bb**	0.527
2019	190	0.014 **m**	0.081	190	0.041**BE**	0.149	190	2.343**ABC**	0.233	173	2.943**aBC**	0.394
2020	61	0.253 **lm**	0.143	61	-1.452**CDE**	0.262	61	-1.609 **bC**	0.409	60	-3.928**AbC**	0.696
Season												
I	245	0.138 **n**	0.055	245	0.018	0.101	245	1.095 **D**	0.157	231	0.743 **c**	0.269
II	410	-0.138 **n**	0.055	410	-0.018	0.101	410	-1.095 **D**	0.157	396	-0.743 **c**	0.269
Farm												
№ 1	211	0.384 **A**	0.082	211	1.714 **FG**	0.149	211	6.315 **EF**	0.234	200	3.385 **DE**	0.404
№ 2	219	0.135 **B**	0.063	219	-0.630 **FH**	0.115	219	-2.392**EH**	0.180	208	-1.733 **D**	0.305
№ 3	225	-0.519**AB**	0.066	225	-1.084 **GH**	0.121	225	-3.924**FH**	0.189	219	-1.652 **E**	0.319
μ	**655**	**4,660 ± 0.054**		**655**	**14,890 ± 0.099**		**655**	**27,861 ± 0.156**		**627**	**56,341 ± 0.261**	

μ - overall LS mean;
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

The results in Table 4 show a higher average daily gain of female lambs born in 2018 to 70 days and 2019 at all ages (P<0.05, P<0.001). Those born in 2020 are presented with negative LS-assessments for the three periods, and the offspring from 2017 gave different deviations from the average in the different periods. Animals born in different seasons are presented with insignificant and without statistically significant differences. Between 30 and 70 days, female offspring born in the spring achieved significantly higher gain (P<0.001). Female lambs from Farm 1 achieved significantly higher gain to 70 days compared to animals from the other two farms (P<0.001). In Farm 2 and Farm 3, the opposite trend was observed. After 70 days, they showed a slightly higher value of the average gain (P<0.05, P<0.01, P<0.001). The average daily gain of Ile de France female lambs in the first month after birth was 0.341 kg, 0.325 kg up to 70 days and 0.143 kg between 70 days and 9 months. Close to our results were those published in the Yearbook of the National Institute of Animal

Husbandry in France, which conducted its research together with INRAe. The Ile de France sheep in their homeland have achieved 0.354 kg average daily gain from 30 to 70 days in 2018 and 0.358 kg in 2019 (Bilan du Contrôle de Performances Ovins Allaitants - Campagne 2019, Institut de l'Elevage/IDELE), INRAe, Races de France). Dimitrov et al. (1987) published lower values of the trait up to 30 days (0.266 kg) for lambs from imported purebred mothers, but during the second period they observed more intensive gain with an increase of 0.313 kg. Ivanova and Raicheva (2017) published data on the average daily gain of Ile de France female lambs from different lineage from 0.208 kg to 0.299 kg, noting that individuals gain more by the 30th day, and twins increase the intensity of growth after 30 to 70 days. Laleva et al. (2020) also reported slightly lower growth values of lambs from the two herds of research institutes at Agricultural Academy, but concluded that offspring could be weaned earlier for meat production.

The analysis of our results confirms the fact that the breed is well adapted in Bulgaria and has retained its potential for high growth intensity at an early age, with a very good average daily gain for lambs.

Table 4. LS-estimates (LSC) of the: year of birth, season and farm effects on the trait average daily gain of female lambs within different growth terms

Term	1 day-30 days			30 days-70 days			70 days-9 months		
	n	LSC	SE	n	LSC	SE	n	LSC	SE
Year of birth									
2017	313	0.039 **lAB**	0.004	313	-0.056 **ABC**	0.004	312	0.011 **alA**	0.002
2018	91	0.016 **l**	0.006	91	0.003 **AD**	0.007	82	-0.002 **a**	0.003
2019	190	0.001 **A**	0.004	190	0.058 **BDE**	0.005	173	0.003 **lB**	0.002
2020	61	-0.057 **B**	0.008	61	-0.004 **CE**	0.009	60	-0.012 **AB**	0.004
Season of birth									
I	245	-0.004	0.003	245	0.027 **F**	0.004	231	-0.002	0.001
II	410	0.004	0.003	410	-0.027 **F**	0.004	396	0.002	0.001
Farm									
№ 1	211	0.044 **CD**	0.005	211	0.115 **GH**	0.006	200	-0.014 **CD**	0.002
№ 2	219	-0.026 **C**	0.004	219	-0.044 **Ga**	0.004	208	0.003 **Cm**	0.001
№ 3	225	-0.019 **D**	0.004	225	-0.071 **Ha**	0.005	219	0.011 **Dm**	0.002
μ	**655**	**0.341 ± 0.003**		**655**	**0.325 ± 0.004**		**627**	**0.143 ± 0.001**	

μ - overall LS mean;
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

CONCLUSIONS

Year of birth had a significant effect on the live weight trait in female Ile de France lambs in all studied ages, with the exception of weight at birth. Season of lambing also affected all ages, excluding weight at 30 days.

The realized gain by periods indicates a significant specific variance depending on the year of birth for all studied ages. The season influenced gain only at 30 to 70 days.

The farm had a highly significant effect on live weight and average daily gain for the selected animals in all ages.

The results for average live weight and average daily gain from birth to 9 months confirmed that the good adaptation of the breed in Bulgaria and the possibility for realization of its potential for high growth intensity at an early age.

ACKNOWLEDGEMENTS

This research work was carried out with the support of Institute of Animal Science – Kostinbrod and also was financed from Project Zh No. 157 of Agricultural Academy - Sofia.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

TRANSGENIC *BOMBYX MORI* AS A BIOTECHNOLOGICAL PLATFORM TO PRODUCE RECOMBINANT PROTEINS

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Abstract

In recent years, the scientific community has been focused on developing feasible platforms to obtain recombinant proteins in order to meet the high demand. There is a wide range of bioreactors that are currently used for this purpose, as bacteria, yeast, mammalian, plant or insect cells. In this regard, decades of dedicated research have shown that the insect biotechnology field is showing the most promising results. In this direction, Bombyx mori exhibits a great potential as a bioreactor to produce target proteins as it possesses various advantages. As expression host, Bombyx mori cells provide optimal post-translational modifications and display a remarkable ability to produce in a short period a great amount of proteins. In this review we discuss progress and we highlight the potential of using transgenic Bombyx mori as a biotechnological platform to obtain both target proteins and to produce enhanced silk fibres. The versatility and the feasibility of transgenic Bombyx mori have been outlined by studies that reported the successful production of human proteins like adiponectin, animal proteins, virus-derived proteins and enhanced silk threads.

Key words: *Bombyx mori, insect biotechnology, recombinant proteins, transgenic silkworms.*

INTRODUCTION

Proteins are complex molecules that have a wide range of applications in various fields, specifically they are important elements for the food, chemical, textile, agriculture and cosmetic industries. Also, due to its diversity, proteins play a key role in the pharmaceutical field and in the medical area. These complex molecules are used as therapeutic proteins and research reagents, but they are also tools used for diagnosis (Dimitrov, 2012; Puetz & Wurm, 2019). Thus, the demand for recombinant proteins is continually increasing, and currently there are major efforts being made by the scientific community to develop feasible biotechnological bioreactors to produce recombinant proteins. In this regard, the insect biotechnology field is showing the most promising results (Chen et al., 2018).

Therefore, there are described various techniques to successfully engineer host's expression system and to successfully extract and purify the target proteins (Schillberg et al., 2019). In this regard, there are various expression systems that have been described and used, such as bacteria, yeast, mammalian, plant or insect cells. The most used host in order to produce recombinant proteins is *Escherichia coli* (*E. coli*), but there is a major throwback, specifically the limited post-translational modifications needed for proteins proper functionality (Nakaya et al., 2020). Even if mammalian cells are the most suitable hosts for recombinant protein production due to its complex processing mechanisms, there are impediments like the high production costs and high risk of contamination (Kollewe & Vilcinskas, 2013).

Insect cells represent ideal bioreactors to produce complex proteins. *Bombyx mori* is one of the most studied insects due to its great economic impact. Fibroin and sericin are the main proteins that are found in silk fibers. Fibroin is synthesized in the posterior silk gland (PSG) and sericin is synthesized in the middle silk gland (MSG) (Xu, 2014).

Silkworms are not only important for sericulture, moreover *Bombyx mori* is a prominent candidate as a bioreactor for recombinant proteins production and represents an important model organism in life sciences

(Chen et al., 2018; Meng et al., 2017). As expression host, *Bombyx mori* cells provide the post-translational modifications required for recombinant proteins structure and functionality. A great advantage owned by *Bombyx mori* as a bioreactor, is the remarkable ability to produce in a short period, a great amount of silk proteins. Another advantage exhibited by silkworms are the numerous genes that are homologous to human genes and the short generation time (Chen et al., 2018).

We herein, by highlighting the pivotal role of *Bombyx mori* in life sciences, review the literature that brings into focus the use of silkworms as a platform to obtain recombinant proteins with an extraordinary impact in the medical, animal sciences and insect biotechnology areas. Furthermore, the use of *Bombyx mori* as a bioreactor exhibits a great step forward for entomology.

PRODUCTION OF FOREIGN PROTEIN IN *BOMBYX MORI*, BY USING A BACULOVIRUS BASED SYSTEM

The first recombinant protein obtained by using *Bombyx mori* as a bioreactor, was the human interferon alpha (IFN-α), that exhibits a great importance for the pharmaceutical industry. For this purpose Maeda et al. (1985) used for the first time *Bombyx mori* nuclear polyhedrosis virus (BmNPV) as an expression system. The gene sequence of IFN-α was driven by the polyhedrin gene promoter, in order to achieve a substantial level of expression. After the expression of recombinant proteins and their secretion in the hemolymph, it was observed that the exogenous proteins are degraded by the cysteine protease. To overcome this obstacle, in order to obtain a higher level of recombinant proteins, another vector which was unable to express the cysteine protease was constructed (Kato et al., 2010).

The first step in the production of recombinant BmNPV is cloning the target gene into the transfer vector. For homologous recombination to take place, there is a second step involved, specifically the co-transfection with the virus DNA into the target cells. The most complicated part of this method is to differentiate and to isolate the recombinant baculoviruses from the ones which have not been transformed (Xiang et al., 2010). However, this process is laborious and time consuming (3-6 months) (Kato et al., 2010).

PRODUCTION OF TRANSGENIC *BOMBYX MORI* GERM-LINE BY USING A PIGGYBAC TRANSPOSON BASED SYSTEM

Since Tamura et al. (2000) reported the development of a complex for stable germline transformation in *Bombyx mori* by using a transposon named piggyBac, the interest of the scientific community has been focused on using *Bombyx mori* as a bioreactor to produce recombinant proteins, emphasizing the key role played by silk gland in production of this type of proteins.

The piggyBac transposon was first isolated from *Trichoplusia ni* (Cabbage looper). Being a movable genetic element, piggyBac can transpose its location between vectors and chromosomes, representing a “cut and paste” tool. PiggyBac as a vector has great benefits, specifically it is safer than a viral vector and it can transpose larger DNA fragments (Zhao et al., 2016). Representing an important tool for genome’s manipulation, piggyBac transposon has been carefully studied and has been shown that it possesses certain features, for example in most cases (98%) it prefers as a site for integration, adenine (A) - timine (T) rich sequences (Li et al., 2013; Yusa, 2015). The main components of piggyBac transposon are the inverted terminal repeats (ITRs), which are located to both ends, and an enzyme named transposase (Li et al., 2013).

Tamura et al. (2000) used a piggyBac system containing the green fluorescent protein (GFP). The gene was placed under the control of *Bombyx mori* cytoplasmic actin gene promoter (*BmA3*). The two elements were placed between the ITRs sequences. A non-autonomous helper plasmid which carried the transposase enzyme, was also used. They reported that in the G1 broods about 2% of individuals have been successfully genetically transformed. After these results were reported, many studies have focused on this topic, thus a wide range of recombinant proteins with applicability in the medical field and beyond, were produced.

SILK GLAND EXPRESSION SYSTEMS IN *BOMBYX MORI*

In the process of producing valuable recombinant proteins by using transgenic *Bombyx mori* as a biotechnological platform, the silk synthesis system is involved. However, the fibroin gene and the sericin gene are involved, being powerfully expressed in the silk gland. Up to now, for the production of recombinant proteins by using *Bombyx mori* as a bioreactor, several silk gland expression systems have been described. Each of these systems has advantages and disadvantages, but the target protein determines which kind of system is going to be used (Xu, 2014). Choosing the right system is the most important step for the recombinant proteins production.

Fibroin L chain gene expression system in *Bombyx mori*

The fibroin L chain gene (*FibL*) expression system was the first system of this type used to express an exogenous protein in the silkworms. When using this kind of expression system, the target proteins are secreted into the lumen of PSG as independent macromolecules. This structure has three main elements, specifically a 5′-flanking sequence, a 3′-flanking sequence, and a partial cDNA sequence of the *FibL* (Xu, 2014).

Tomita et al. (2003) applied *FibL* gene expression system for the production of human collagen in *Bombyx mori*. Xue et al. (2012) also used this system to express in *Bombyx mori* a hematopoietic growth factor, named human granulocyte-macrophage colony stimulating factor. In order to be secreted into the PSG lumen, the recombinant protein has to be linked with the fibroin H chain *(FibH)*. The transgene produced in *Bombyx mori* cannot compete with the endogenous normal *FibL* in the process of S-S linking with the *FibH*, since the normal *FibL* chain has higher affinity for the *FibH* chain. As the unmodified *FibL* has a stronger affinity for the *FibH*, there is a lack of the disulfide bond between the target protein and the *FibH*, that explains the low expression level of recombinant proteins (Tatemastu, 2012). To overcome this impediment, to obtain a higher production of recombinant proteins, Inoue et al. (2005) obtained mutant silkworms which did not have a full endogenous *FibL* gene sequence.

Fibroin H chain gene expression system in *Bombyx mori*

The expression system that involves the *FibH* gene is the most used system of this kind in *Bombyx mori* for the production of improved silk, needed for medical purposes. The most important advantage of this kind of system is the promoter, the *FibH* promoter has a stronger activity in *Bombyx mori* than the *FibL* promoter (Tatemastu, 2012). In this category of expression structures are included three systems R1, R2 and R3 (Xu, 2014). The last one is the most efficient expression system and its structure consists of *FibH* promoter and the N- and C-terminal ends of the *FibH* (Tatemastu, 2012). Also, when using this type of system, the recombinant proteins are secreted in the lumen of PSG as independent proteins. Even if the highest level of fibroin is found in the PSG, it is easier to extract and purify the target proteins from the MSG (Xu, 2014).

Teulé et al. (2012) used the *FibH* gene expression system to improve the mechanical properties of the silk thread, specifically they genetically manipulated *Bombyx mori* to produce silk threads containing sequences of spider silk proteins. The transgenic *Bombyx mori* lines produced silk fibers more resistant than the fiber produced by the untransformed silkworm lines. They observed that the silk threads were not only tougher than the silkworm fibers, but even tougher than the original spider silk fibers.

Sericin-1 expression systems in *Bombyx mori*

The three genes that are involved in the sericin synthesis are *ser1*, *ser2* and *ser3*. The proteins encoded by the first and the last genes shape the cocoon's sericin layer. The *ser2* gene encodes proteins which have been shown that are linked with larval silk (Kunz et al., 2016). The promoter activity of *ser1*, *ser2* and *ser3* genes was analyzed by Tatematsu et al., (2010) as the request for the production of recombinant proteins using feasible bioreactors continues to expand.

In order to examine the promoters activity, they used the binary GAL4/UAS expression system for the expression of EGFP. Using the *ser1* gene promoter, strong activity was observed in the PSG and MSG. In the MSG, the *ser3* upstream region showed average activity, but the *ser2* promoter did not show activity in none of the regions of the silk gland.
To increase the expression level of recombinant proteins the promoter's activity has to be improved. Thus, this type of expression system involves the use of an enhancer, specifically *hr3* (baculovirus-derived) and also implicates a trans-regulator, IE1 (Tomita et al., 2007).

SILK FIBERS WITH ENHANCED PROPERTIES OBTAINED BY USING TRANSGENIC *BOMBYX MORI*

Using transgenic *Bombyx mori* to create silk fibers with improved cell proliferation activity

A key role in tissue regeneration is played by the fibroblast growth factors. An important member of the fibroblast growth factors family is the basic fibroblast growth factor (FGF2) which is encoded by *FGF2* gene and for the first time it was isolated from the pituitary gland. FGF2 is involved in cell growth, differentiation, being an important element for tissue regeneration, including skin, muscle, cartilages etc., but also several studies have shown that it has an important role in postnatal neurogenesis, dendritic plasticity (Coffin et al., 2018; Simard et al., 2018; Yun et al., 2010). The transforming growth factor β1 (TGF- β1) is a cytokine which plays a pivotal role in the healing process, also is essential for the maturation of lymphocytes, neutrophils and macrophages (Lodyga & Hinz, 2020).
Wang et al. (2019) used transgenic *Bombyx mori* to obtain silk thread containing FGF2 and TGF- β1, in order to improve the silk fibers for medical uses, specifically for improving the cell proliferation and the anti-inflammatory activity. To construct the transgenic vector, they used the *ser1* promoter, nuclear polyhedrosis virus enhancer *hr3* and the 3′-UTR of *ser1*. GSG-P2A self-cleaving peptide was used to associate the target genes sequences with the *Bombyx mori* codon preference, and also the 3xp3-DsRed-SV40 cassette was integrated. Microinjection is the technique which has been used for delivering the transgenic vector into non-diapausing eggs. They successfully genetically engineered *Bombyx mori* to co-express the recombinant proteins in the silk fibers and MSG.
Acidic fibroblast growth factors (FGF1s) are heparin binding macromolecules and play a major role in cell growth and proliferation. Aiming to increase cell's proliferation activity, there was reported the integration of FGF1s in the *Bombyx mori*'s silk threads (Davis et al., 2018; Kerr et al., 2019).
Another protein involved in cell proliferation, with numerous applications in the medical area, is the human connective tissue growth factor (CTGF) (Wang et al., 2020). It plays a key role in extracellular matrix remodeling, being a cysteine-rich protein (Tsai et al., 2018; Wang et al., 2020). Due to its role in the medical field, specifically in wound healing and implantation, there is a need to produce this protein by using feasible bioreactors. Wang et al. (2020) used two modified *Bombyx mori* strains to produce recombinant CTGF. One of them carried *CTGF-8ht* gene, and the other one contained the *pepCTGF-8ht* gene (transdermal peptide), also an enhanced His-tag was included in both strains. *Bombyx mori* was successfully used as a bioreactor as the target proteins were both expressed in the silk gland and cocoon. In this case, an advantage of using silkworms as a bioreactor was the ease of extraction and purification process. Comparing the proliferation activity of the two recombinant proteins, *pepCTGF-8ht* displayed a better activity.

Using transgenic *Bombyx mori* to produce silk fiber with improved mechanical properties

One of the biopolymers that have superior mechanical features is the spider silk. The most important advantages of spider silk are the strength and the elasticity. These features make it a great candidate for medical applications, but farming the spiders encounters certain obstacles, thus the large-scale production must be done by using a feasible bioreactor (Tokareva et al., 2013; Xu et al., 2018). In order to produce spider fibers, numerous

bioreactors were used (bacteria, yeast, etc.), but the expressed proteins did not meet expectations (Xu et al., 2018). It has been shown that due to the most important spider silk macromolecule, specifically the major ampullate silk protein encoded by *MaSp* gene, the silk fiber exhibits superior mechanical features. This protein has a highly repetitive structure composed of glycine and alanine rich-regions (Santos-Pinto et al., 2016; Jun Xu et al., 2018). Being able to produce proteins that have highly repeated blocks in their structure, *Bombyx mori* is a suitable bioreactor to produce silk containing spider silk polypeptides.

Kuwana et al. (2014) obtained high-toughness silk by using transgenic *Bombyx mori*. They cloned a spider dragline protein fragment (SpA) and for the recombinant protein expression, the *FibH* gene expression system was applied. Along with SpA, the enhanced green fluorescent protein (EGFP) was placed between the N- and C- terminal ends of the *FilH.*

Xu et al. (2018) applied the TALEN-mediated HDR technology for editing *Bombyx mori* to replace *FibH* with *MaSp1.* They performed this study to obtain mass-production of spider silk due to its great mechanical properties. The results showed that the transgenic silk threads did not have the same strength as the original silk fibers, but the elasticity was improved.

In order to increase the tenacity and the extension of silk threads, another study was performed by Wang et al. (2015). Aiming to reduce the calcium content in silk and to increase the α-helix and β-sheet structures, they successfully transformed silk threads not by changing the gene sequence but by overexpression ion-transporting proteins.

Using transgenic *Bombyx mori* to improve cell-adhesive properties of silk fibers

Collagen and fibronectin are the most used proteins as layers for the surface of cell culture plates. Due to low availability of the two proteins, silk of *Bombyx mori* is a feasible alternative for this purpose (Yanagisawa et al., 2007). Though, the adhesive feature of silk fibers is not as strong as adhesive ability of collagen and fibronectin. It has been shown that using fibroin for coating the surface of fibroblast or endothelial cells cultures, the adhesion level was weak (Jacobsen et al., 2017). In order to increase the level of adhesion when using the silk fibers on the surface of cell cultures plates, Yanagisawa et al. (2007), inserted into the *Bombyx mori*'s genome, partial sequences of collagen and fibronectin. The results showed that the transgenic silk fibers which contained a fragment of recombinant fibronectin had stronger adhesion activity than the ones which had the partial sequence of collagen.

Enhanced silk with antimicrobial and anti-inflammatory activities by using transgenic silkworms

In order to obtain silk with enhanced properties, particularly with antimicrobial and anti-inflammatory activities, Xu et al. (2019) genetically engineered silkworms to produce recombinant human lactoferrin. This nutrient is a cationic glycosylated protein and has two main lobes, each one owning an iron-binding domain. It is found mainly in mammalian milk, but it is also found in other exocrine secretions like saliva, tears or serum (Kell et al., 2020; Sill et al., 2016). Lactoferrin plays a critical role in the innate immune response, owning antibacterial, antifungal and antiviral properties. Even if lactoferrin is well-known for the bactericidal activity, it also has anti-inflammatory and anti-carcinogenic effects (Kell et al., 2020; Xu et al., 2019).

Due to its great therapeutic activities, the demand for lactoferrin is continually increasing. Xu et al. (2019) used the *Ser-1* expression system to obtain human lactoferrin in silkworms. Their data showed that the level expression of lactoferrin is influenced by the transgene's insertion position. The results also confirmed the inhibitory effect of recombinant human lactoferrin. However, their findings showed that *Bombyx mori* is a promising candidate and a cost-effective bioreactor to produce recombinant proteins.

Gloverin2 (Glv2) is an antibacterial glycine-rich protein, being a key player in the lepidopteran insects innate immune response. Even if the silk threads from *Bombyx mori*'s cocoons exhibit antimicrobial activity, it is not able to combat the infections, thus it cannot be

used for medical purposes. Wang et al., (2019) developed transgenic silkworms by overexpressing the Glv2 protein. They reported the success of developing silk threads that exhibit an increased antimicrobial activity against various species of bacteria and fungi, by using transgenic *Bombyx mori*.

RECOMBINANT PROTEINS OBTAINED BY USING TRANSGENIC *BOMBYX MORI* AS A BIOREACTOR

Recombinant animal proteins

Nakaya et al. (2020) used the expression system of *Bombyx mori* to obtain a nuclear receptor, specifically the thyroid hormone receptor. This family of receptors has two main elements, respectively TRα1 and TRβ1. In the process of fulfilling the role of thyroid hormone function, the two receptors play a crucial role, being ligand-dependent transcription elements. However, these receptors moderate the expression of certain genes and control several main processes as metabolism or growth (Anyetei-Anum et al., 2018). The authors successfully obtained recombinant mice TRβ1 by using *Bombyx mori* as a bioreactor. To obtain the target receptor they used the piggyBac vector and the *FibH* gene promoter that were put together with glutathione S-transferase from *E. coli*. The entire fragment was controlled by the GAL4/UAS system and the EGFP was used to confirm subsequently the success of transformation. However, another transgenic vector was constructed in order to obtain PSG specific expression (Nakaya et al., 2020).

The main component and the most abundant protein of royal jelly is the major royal jelly protein-1 (MRJP1). MRJP1 received great attention from the scientific community due to its therapeutic properties in humans (Tian et al., 2018).

In the same direction, You et al. (2017) used transgenic silkworms to produce this glycoprotein. They successfully used the *FibL* expression system to secrete recombinant MRJP1 into the cocoons. In terms of post-translational modification, which is the main reason for not using bacteria or yeast as bioreactors, their data shown that glycosylation of exogenous protein occurred in *Bombyx mori*, highlighting the feasibility of using the transgenic silkworms to produce target proteins.

Antimicrobial peptides (AMPs) are indispensable proteins which are implicated in host's innate immune response to bacteria, fungi or viruses. AMPs are included in numerous organisms like plants or animals (Lei et al., 2019). It has been shown that the black soldier fly (BSF) owns a great potential to live in microbe-rich, hostile environments. However, BSF is currently studied in order to reduce the waste amount due to its extraordinary ability to transform the waste into valuable biomass (Mertenat et al., 2019). Thus, owning this great ability, BFS is one of the most important sources of AMPs (Moretta et al., 2020). Another direction of approach and use of transgenic silkworms, was reported by Xu et al. (2020). They aimed to use transgenic *Bombyx mori* to reduce the incidence of pathogen infections, thus to help the sericulture industry. By using a piggyBac vector, the authors introduced an AMP cassette derived from the BSF. In the cassette named HiAMP4516, the authors incorporated three AMPs, specifically *Hidefensin-1, Hidiptericin-1* and *HiCG13551*. Their results proved that *Bombyx mori*'s susceptibility to bacterial pathogen infection could be reduced by using genetic engineering.

Iizuka et al. (2009) reported the successful use of *Bombyx mori* as a bioreactor to produce recombinant mouse monoclonal antibodies (mAbs) in the cocoons. The use of mAbs is a promising therapeutic method to prevent the infectious disease (Jahanshahlu & Rezaei, 2020). They highlighted the ease of mAbs extraction and purification, thus the feasibility of using *Bombyx mori* as a bioreactor to produce recombinant mouse mAbs (Iizuka et al., 2009).

In addition to mAbs role in the prevention of the infection diseases, the use of recombinant mAbs is one of the most promising methods against cancer. As a bioreactor, Chinese hamster ovary (CHO) cells are the most used cells to obtain recombinant mAbs.

Even if there is a wide range of therapeutic mAbs that have been approved for medical purposes, the process of obtaining therapeutic

mAbs by using this type of cells is not cost effective. Due to the high costs involved in obtaining therapeutic mAbs, Tada et al. (2015) used transgenic *Bombyx mori* to produce chimeric human-mouse anti-CD20 mAbs in order to provide successful target therapy for different types of cancers. In this study the expression level of anti-CD20 mABs produced by using transgenic silkworms was compared with the level of expression observed by using CHO cells. The mAbs used contained an analogous sequence to rituximab, that is one of the most used agents against cancer. Their data revealed a unique feature owned by recombinant mAbs obtained by using transgenic *Bombyx mori*, specifically the mAbs containing N-glycan structures. Compared with the antibody-dependent cellular cytotoxicity level observed in the mAbs produced by using Cho cells, the level observed in mABs derived from transgenic *Bombyx mori*, was higher. Comparing the complement-dependent cytotoxicity activity observed in the mAbs obtained by using both type of cells, the mABs derived from transgenic silkworms present a lower activity that the mAbs obtained by using CHO cells (Aoyama et al., 2018; Tada et al., 2015).

Recombinant human proteins

Vascular endothelial growth factors (VEGFs) play a crucial role in angiogenesis. Angiogenesis represents a critical step in cancer development, precisely in the process of changing the cancer state from benign to malignant. The VEGFs effect on tumor cells results in protecting them from apoptosis (Shibuya, 2011). There is a massive demand for VEGFs due to its applicability in the medical field, specifically the scientific community is focused on developing strategies to inhibit the VEGF in order to treat cancer. Another reason besides the research for developing anti-cancer treatments, is the therapeutic induced angiogenesis by VEGF. On this purpose Zhang et al. (2019) constructed transgenic silkworms that expressed recombinant VEGF165 in MSG and secreted it into the cocoons. This study underlines the feasibility of using transgenic silkworms to produce target recombinant proteins.

Another research has been focused on using transgenic silkworms to evaluate the response of target human protein to certain drugs. The authors developed transgenic *Bombyx mori* that synthetized the human insulin receptor (hIR) (Matsumoto et al., 2014). Insulin is a pancreatic hormone that has a crucial function in the organism due to its role in the metabolism of glucose and lipids. Glucose homeostasis depends on the insulin level; the hormone controls the conversion of glucose into glycogen. To regulate the metabolism, the insulin must bind to its specific receptor, hIR (Hall et al., 2020). Matsumoto et al. (2014) obtained recombinant hIR by using transgenic silkworms and demonstrated its ability to reduce the hemolymph sugar level. Their data highlighted the similarity between the silkworms and humans, in terms of drug pharmacokinetics, however, the transgenic lines could be used to examine the curative outcome of hIR agonists.

Adiponectin is a peptide secreted by adipocytes being an important factor for glucose and lipid metabolism. However, low concentration level of adiponectin is associated with obesity related disorders, like diabetes or various cardiovascular affections. This protein has an important insulin-sensitizing activity and represents a pivotal player in the process of decreasing the insulin resistance, in order to combat type 2 diabetes (Achari & Jain, 2017; Luo et al., 2020). Being a potential versatile therapeutic agent there is a high demand for human adiponectin in the medical area. Shin et al. (2014), in an effort to combat the obesity rate, used *Bombyx mori* as a bioreactor to obtain human adiponectin. Their findings showed that the target recombinant protein was successfully produced in silkworms and the recombinant adiponectin is a promising future therapy for type 2 diabetes patients.

Recombinant virus-derived proteins

Hepatitis B virus (HBV) causes liver infection that leads to cirrhosis or hepatocellular carcinoma. In order to develop a new effective vaccine against Hepatitis B virus, Abdurakhmanov et al. (2019) used *Bombyx mori* to obtain recombinant PreS2-S protein. This protein is a family member of a surface

antigen of HBV. Their aim was to develop a method that is cost effective and not time-consuming to obtain the PreS2-S protein in silkworm larvae. The recombinant PreS2-S protein obtained by using BmNPV as an expression vector, is a potential candidate for developing a new vaccine against HBV.

The use of recombinant subunit vaccines is a promising approach to avoid the safety concerns of using cell-culture based vaccines. The recombinant subunit vaccines do not involve the use of a virus but the use of virus-like particles (VLPs). The transgenic silkworms were used as a platform to produce recombinant target protein by Deo et al. (2011), specifically they used this type of bioreactor to obtain Rous sarcoma virus-gag virus-like particles. Their data highlight the feasibility of using transgenic silkworms to obtain VLPs in order to produce vaccines.

CONCLUSIONS

Although there are numerous expression platforms that are used to produce target proteins that own a great impact for animal science area but also for the human health and beyond, transgenic *Bombyx mori* is one of the most promising candidates in this regard. This expression system is feasible due to *Bombyx mori*'s remarkable ability to satisfy the extensive post-translational modification required for recombinant proteins structure and functionality. There are various studies in which *Bombyx mori* was manipulated to enhance the silk threads quality for medical purposes. Numerous studies reported the success of developing silk threads that possessed improved cell-adhesive properties, better mechanical features or silk fibers that owned antimicrobial and anti-inflammatory activities. These findings highlight the key role of *Bombyx mori* in obtaining biomaterials with enhanced properties. Furthermore, there is a broad variety of recombinant proteins with clinical importance that have been developed by using silkworms as expression hosts. The versatility and the feasibility of using this type of bioreactor have been highlighted by the studies that reported the successful production of human proteins, animal proteins and virus-derived proteins. This review highlights the feasibility and the importance of *Bombyx mori* as a powerful biotechnological platform for large-scale production of recombinant proteins.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

COMPARATIVE RESEARCH CONCERNING HARDY–WEINBERG EQUILIBRIUM IN FOUR STATISTICAL SWINE POPULATIONS

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Abstract

The state of genetic balance in one or more loci, no matter if it concerns the loci involved in genetic determinism of quality or quantity traits, describes an ideal state, being influenced by natural selection, non-random mating, gene flow and genetic drift. The Hardy-Weinberg equilibrium trial presents a significant importance because any deviation from it reveals the existence of some disturbing, restrictive factors. Thus, in this direction, there were researched four swine statistical livestock, taking into account the aggregate genotype derived from prealbumins, transferrins and serum amylases. The significant distribution of the individuals which present the genotypical combination $Pa^BPa^B/Tf^BTf^B/Am^BAm^B$ -(Landrace - 39.5%; Large White - 23.7%; Synthetic Breed P 2000 - 22.7%; Synthetic 345 - 31.9%) was characteristic of the four researched samples. Out of the 27 possible combinations in the three loci, there weren't identified individuals with the aggregate genotypes in any sample: $Pa^APa^A/Tf^ATf^A/Am^AAm^A$; $Pa^APa^A/Tf^ATf^A/Am^AAm^B$; $Pa^APa^A/Tf^ATf^B/Am^AAm^A$; $Pa^APa^A/Tf^BTf^B/Am^AAm^A$; $Pa^APa^B/Tf^ATf^A/Am^AAm^A$; $Pa^APa^B/Tf^ATf^B/Am^AAm^A$. In order to estimate the balance state, it was calculated the determinant of the gamete matrix. The result of the genetic state Hardy Weinberg trial, simultaneously for the three loci ($g_{11}g_{33}g_{17}g_{39} = g_{31}g_{13}g_{37}g_{19}$), emphasized the fact that none of the livestock is in genetic balance, as it follows: Landrace (0.0000215 ≠ 0.0000067), Large White (0.000039 ≠ 0.000041), Synthetic Breed P 2000 (0.000055 ≠ 0.000025), Synthetic Breed 345 (0.000004 ≠ 0.000030). This shortage of balance was caused by an indirect selection, with a higher coefficient of some genotypic combinations in comparison with the others.

Key words*: Hardy-Weinberg equilibrium, gamete matrix, genotip matrix, pig populations.*

INTRODUCTION

Protein polymorphism manifests itself from birth and, with some exceptions, it is constantly maintained throughout the life of the animal (Bacila V. et al., 2011). It is possible to use it as an early selection tool for high-value breeders since some protein fractions can be correlated with some economic traits (Drăgotoiu T., 2005).

Also, the importance of knowing proteins genetic determinism (protein fractions) lies in the application of information in this field, in the following directions:

- Clarification of cases of uncertain origin since biochemical systems provide information on the specific genetic material of each individual (Rebedea, 1991);
- Establishing paternity (Rebedea, 1992);
- Decoding the genetic mechanisms responsible for the existence of polymorphism at the level of biochemical structures (Juneja, 1988);
- Establishing phylogeny and kinship among breeds, based on the frequencies of genes that determine different biochemical structures (Defta, 2001), structures characteristic to certain breeds and population. "Changes in allele frequencies over time can indicate that genetic drift is occurring or that new mutations have been introduced into the population" (www.nature.com).
- Being major genes, their manifestation is discontinuous, thus, they can be used as marker genes, establishing correlations among them and quantitative traits loci (QTL). "Quantitative trait locus (QTL) analysis is a statistical method that links two types of information - phenotypic data (trait measurements) and genotypic data (usually molecular markers) - in an attempt to explain the genetic basis of variation in complex traits" (Miles, 2008).

MATERIALS AND METHODS

a. **Material and laboratory methods**

To determine the types of serum proteins, i.e. prealbumins, transferins and amylases, there were taken blood samples from 290 heads of the following breeds: Landrace (76), Great White (76) and synthetic lines P 2000 (66) and 345 (72).

The subjects that constituted the source of blood sampling were chosen at random.

The vertical electrophoresis technique was used to determine the types of *transferins and prealbumins* in the study individuals. (Smithies, 1955; Costache, 2004), using polyacrylamide as migration support.

To emphasize the types of *serum amylases*, electrophoresis in starch gel was used as a working method in a discontinuous system of swabs (Meriaux, 1992).

b. **Data processing methods**

For the three loci, the number of genotypic combinations which may result is 27.

Thus, the categories frequencies of aggregate genotypes were organized into a matrix, with three rows and nine columns.

Considering account simultaneously the three loci, each one, with simple allelism, can result in eight categories of gametes, the frequency of which is organized in a matrix of the type:

$A_1B_1C_1$	$A_1B_1C_2$	$A_1B_2C_1$	$A_1B_2C_2$
$A_2B_1C_1$	$A_2B_1C_2$	$A_2B_2C_1$	$A_2B_2C_2$

respectively $g = \begin{bmatrix} g_{11} & g_{13} & g_{17} & g_{19} \\ g_{31} & g_{33} & g_{37} & g_{39} \end{bmatrix}$

Knowing the frequencies of the genotype categories, the population's gamet background was also determined, knowing that, for example, the category of gametes of type $A_1B_1C_1$ will represent 100% of the gametes produced by genotype $A_1A_1/B_1B_1/C_1C_1$; 50% out of the gametes produced by $A_1A_1/B_1B_1/C_1C_2$, $A_1A_1/B_1B_2/C_1C1$ and $A_1A_2/B_1B_1/C_1C_1$ genotypes; 25% out of the gametes produced by the $A_1A_1/B_1B_2/C_1C_2$ genotypes; $A_1A_2/B_1B_1/C_1C_2$ and $A_1A_2/B_1B_2/C_1C_1$ and 12.5% out of the gametes produced by the genotype $A_1A_2/B_1B_2/C_1C_2$.

$A_1B_1C_1 = g_{11} = G_{11} + \frac{1}{2}(G_{12} + G_{14} + G_{21}) + \frac{1}{4}(G_{15} + G_{22} + G_{24}) + \frac{1}{8}G_{25}$

$A_1B_1C_2 = g_{13} = G_{13} + \frac{1}{2}(G_{12} + G_{16} + G_{23}) + \frac{1}{4}(G_{15} + G_{22} + G_{26}) + \frac{1}{8}G_{25}$

$A_1B_2C_1 = g_{17} = G_{17} + \frac{1}{2}(G_{14} + G_{18} + G_{27}) + \frac{1}{4}(G_{15} + G_{24} + G_{28}) + \frac{1}{8}G_{25}$

$A_1B_2C_2 = g_{19} = G_{19} + \frac{1}{2}(G_{16} + G_{18} + G_{29}) + \frac{1}{4}(G_{15} + G_{26} + G_{28}) + \frac{1}{8}G_{25}$

$A_2B_1C_1 = g_{31} = G_{31} + \frac{1}{2}(G_{21} + G_{32} + G_{34}) + \frac{1}{4}(G_{22} + G_{24} + G_{35}) + \frac{1}{8}G_{25}$

$A_2B_1C_2 = g_{33} = G_{33} + \frac{1}{2}(G_{23} + G_{32} + G_{36}) + \frac{1}{4}(G_{22} + G_{26} + G_{35}) + \frac{1}{8}G_{25}$

$A_2B_2C_1 = g_{37} = G_{37} + \frac{1}{2}(G_{27} + G_{34} + G_{38}) + \frac{1}{4}(G_{24} + G_{28} + G_{35}) + \frac{1}{8}G_{25}$

$A_2B_2C_2 = g_{39} = G_{39} + \frac{1}{2}(G_{29} + G_{36} + G_{38}) + \frac{1}{4}(G_{26} + G_{28} + G_{35}) + \frac{1}{8}G_{25}$

Appropriate situation for three places

$A_1A_1B_1B_1C_1C_1$	$A_1A_1B_1B_1C_1C_2$	$A_1A_1B_1B_1C_2C_2$	$A_1A_1B_1B_2C_1C_1$	$A_1A_1B_1B_2C_1C_2$	$A_1A_1B_1B_2C_2C_2$	$A_1A_1B_2B_2C_1C_1$	$A_1A_1B_2B_2C_1C_2$	$A_1A_1B_2B_2C_2C_2$
$\mathbf{p^2r^2t^2}$	$\mathbf{p^2r^2tu}$	$\mathbf{p^2r^2u^2}$	$\mathbf{p^2rst^2}$	$\mathbf{p^2rstu}$	$\mathbf{p^2rsu^2}$	$\mathbf{p^2s^2t^2}$	$\mathbf{p^2s^2tu}$	$\mathbf{p^2s^2u^2}$
$A_1A_2B_1B_1C_1C_1$	$A_1A_2B_1B_1C_1C_2$	$A_1A_2B_1B_1C_2C_2$	$A_1A_2B_1B_2C_1C_1$	$A_1A_2B_1B_2C_1C_2$	$A_1A_2B_1B_2C_2C_2$	$A_1A_2B_2B_2C_1C_1$	$A_1A_2B_2B_2C_1C_2$	$A_1A_2B_2B_2C_2C_2$
$\mathbf{pqr^2t^2}$	$\mathbf{pqr^2tu}$	$\mathbf{pqr^2u^2}$	$\mathbf{pqrst^2}$	$\mathbf{pqrstu}$	$\mathbf{pqrsu^2}$	$\mathbf{pqs^2t^2}$	$\mathbf{pqs^2tu}$	$\mathbf{pqs^2u^2}$
$A_2A_2B_1B_1C_1C_1$	$A_2A_2B_1B_1C_1C_2$	$A_2A_2B_1B_1C_2C_2$	$A_2A_2B_1B_2C_1C_1$	$A_2A_2B_1B_2C_1C_2$	$A_2A_2B_1B_2C_2C_2$	$A_2A_2B_2B_2C_1C_1$	$A_2A_2B_2B_2C_1C_2$	$A_2A_2B_2B_2C_2C_2$
$\mathbf{q^2r^2t^2}$	$\mathbf{q^2r^2tu}$	$\mathbf{q^2r^2u^2}$	$\mathbf{q^2rst^2}$	$\mathbf{q^2rstu}$	$\mathbf{q^2rsu^2}$	$\mathbf{q^2s^2t^2}$	$\mathbf{q^2s^2tu}$	$\mathbf{q^2s^2u^2}$

where, p= the frequency of the A_1 gene; respectively Pa^A

q= the frequency of the A_2 gene; respectively Pa^B

r= the frequency of the B_1 gene; respectively Tf^A

s= the frequency of the B_2 gene; respectively Tf^B

t= the frequency of the C_1 gene; respectively Am^A

u= the frequency of the C_2 gene; respectively Am^B

and the genotypic matrix

$$G = \begin{bmatrix} G_{11} & G_{12} & G_{13} & G_{14} & G_{15} & G_{16} & G_{17} & G_{18} & G_{19} \\ G_{21} & G_{22} & G_{23} & G_{24} & G_{25} & G_{26} & G_{27} & G_{28} & G_{29} \\ G_{31} & G_{32} & G_{33} & G_{34} & G_{35} & G_{36} & G_{37} & G_{38} & G_{39} \end{bmatrix}$$

For three loci, the gametes frequency is the following: *prt* for $A_1B_1C_1$, *pru* for $A_1B_1C_2$, *pst* for $A_1B_2C_1$, *psu* for $A_1B_2C_2$, *qrt* for $A_2B_1C_1$, *qru* for $A_2B_1C_2$, *qst* for $A_2B_2C_1$, *qsu* for $A_2B_2C_2$. Concerning the situation coming from three loci, the equilibrium condition is reached when the genetic matrix:

$$g = \begin{bmatrix} g_{11} & g_{13} & g_{17} & g_{19} \\ g_{31} & g_{33} & g_{37} & g_{39} \end{bmatrix}$$ achieves the equality $g_{11}\, g_{33}\, g_{17}\, g_{39} = g_{31}\, g_{13}\, g_{37}\, g_{19}$

RESULTS AND DISCUSSIONS

Comparative analysis of the four samples

With the methods described above, there were identified the genotypes characteristic of the four study swine population (Table 1).

Table 1. Genetical structure of the four samples according to aggregate genotypes of prealbumins, transferins and serum amylases

Genotypical combination	Breed							
	Landrace		Great White		LP 2000		LS 345	
	n	%	n	%	n	%	n	%
$Pa^APa^A/Tf^ATf^A/Am^AAm^A$	-	0	-	0	-	0	-	0
$Pa^APa^A/Tf^ATf^A/Am^AAm^B$	-	0	-	0	-	0	-	0
$Pa^APa^A/Tf^ATf^A/Am^BAm^B$	-	0	-	0	1	1.5	-	0
$Pa^APa^A/Tf^ATf^B/Am^AAm^A$	-	0	-	0	-	0	-	0
$Pa^APa^A/Tf^ATf^B/Am^AAm^B$	2	2.6	2	2.6	-	0	1	1.4
$Pa^APa^A/Tf^ATf^B/Am^BAm^B$	-	0	-	0	4	6.1	2	2.8
$Pa^APa^A/Tf^BTf^B/Am^AAm^A$	-	0	-	0	-	0	-	0
$Pa^APa^A/Tf^BTf^B/Am^AAm^B$	-	0	-	0	-	0	1	1.4
$Pa^APa^A/Tf^BTf^B/Am^BAm^B$	3	3.9	-	0	8	12.1	5	6.9
$Pa^APa^B/Tf^ATf^A/Am^AAm^A$	-	0	-	0	-	0	-	0
$Pa^APa^B/Tf^ATf^A/Am^AAm^B$	-	0	4	5.3	1	1.5	-	0
$Pa^APa^B/Tf^ATf^A/Am^BAm^B$	-	0	1	1.3	-	0	-	0
$Pa^APa^B/Tf^ATf^B/Am^AAm^A$	-	0	-	0	-	0	-	0
$Pa^APa^B/Tf^ATf^B/Am^AAm^B$	2	2.6	3	4	4	6.1	-	0
$Pa^APa^B/Tf^ATf^B/Am^BAm^B$	3	4	3	3.9	-	0	6	8.3
$Pa^APa^B/Tf^BTf^B/Am^AAm^A$	3	4	1	1.3	5	7.6	1	1.4
$Pa^APa^B/Tf^BTf^B/Am^AAm^B$	1	1.3	5	6.6	9	13.6	2	2.8
$Pa^APa^B/Tf^BTf^B/Am^BAm^B$	9	11.8	10	13.2	2	3	12	16.7
$Pa^BPa^B/Tf^ATf^A/Am^AAm^A$	-	0	1	1.2	-	0	1	1.4
$Pa^BPa^B/Tf^ATf^A/Am^AAm^B$	1	1.4	-	0	2	3	3	4.2
$Pa^BPa^B/Tf^ATf^A/Am^BAm^B$	2	2.6	2	2.7	4	6.1	-	0
$Pa^BPa^B/Tf^ATf^B/Am^AAm^A$	-	0	2	2.7	-	0	-	0
$Pa^BPa^B/Tf^ATf^B/Am^AAm^B$	6	7.9	7	9.2	-	0	3	4.2
$Pa^BPa^B/Tf^ATf^B/Am^BAm^B$	7	9.2	7	9.2	11	16.7	4	5.5
$Pa^BPa^B/Tf^BTf^B/Am^AAm^A$	-	0	2	2.6	-	0	1	1.4
$Pa^BPa^B/Tf^BTf^B/Am^AAm^B$	7	9.2	8	10.5	-	0	7	9.7
$Pa^BPa^B/Tf^BTf^B/Am^BAm^B$	30	39.5	18	23.7	15	22.7	23	31.9

A common point in the four samples studied is the high proportion of individuals with the $Pa^BPa^B/Tf^BTf^B/Am^BAm^B$ genotypic combination. The highest frequency for this genotypic combination category was determined in the Landrace sample, 16.8% higher than that determined in the LP 2000 sample.

From the data presented in Table 1 one can remark that there are differences among the genetic structures of the four samples, mainly by the absence of different categories with varied genotypic combinations in the respective samples. Thus, from the 27 possible genotypic combinations among the three loci, the Landrace breed population counts 13, and the Great White breed population 16. The sample belonging to the Synthetic Line P 2000 is distinguished by the smallest number of genotypic combination categories, respectively 12. Only 15 categories of genotypic combinations are found in the sample within the Synthetic Line LS 345.

Also, the frequency of the genotype categories found in each of the four samples was different (Table 1).

One doesn't find individuals with the genotypic combinations $IPa^APa^A/Tf^ATf^A/Am^AAm^A$ and $Pa^APa^A/Tf^ATf^A/Am^AAm^B$ in any of the samples, as a consequence of the small share that individuals with type A of transferin had in each of the studied samples.

Equilibrium state estimation

A. *Landrace sample*

The genetic structure setting up allowed the estimation of the equilibrium state for the Landrace sample.

The frequencies of the twenty-seven categories of possible genotypic combinations among the three studied loci were organized in a matrix with the following structure:

$$G = \begin{bmatrix} 0 & 0 & 0 & 0 & 0.026 & 0 & 0 & 0 & 0.039 \\ 0 & 0 & 0 & 0 & 0.026 & 0.040 & 0.040 & 0.013 & 0.118 \\ 0 & 0.014 & 0.026 & 0 & 0.079 & 0.092 & 0 & 0.092 & 0.395 \end{bmatrix}$$

Knowing the frequencies of the genotypes categories, the population's gamete background was also determined. The frequencies of the gamete categories were structured as a matrix, as it follows:

$$g = \begin{bmatrix} 0.010 & 0.020 & 0.033 & 0.121 \\ 0.030 & 0.112 & 0.092 & 0.582 \end{bmatrix}$$

In the case we studied, it wasn't found any genetic equilibrium revealed by the relationship:

$$g_{11} \times g_{33} \times g_{17} \times g_{39} \neq g_{31} \times g_{13} \times g_{37} \times g_{19}$$

respectively:

$$0.010 \times 0.112 \times 0.033 \times 0.582 \neq 0.030 \times 0.020 \times 0.092 \times 0.121$$

$$0.0000215 \neq 0.0000067$$

B. *Great White sample*

The matrix structure according to the frequencies of the 27 aggregate genotypic combinations which can result from the three loci is:

$$G = \begin{bmatrix} 0 & 0 & 0 & 0 & 0.026 & 0 & 0 & 0 & 0 \\ 0 & 0.053 & 0.013 & 0 & 0.040 & 0.039 & 0.013 & 0.066 & 0.132 \\ 0.012 & 0 & 0.027 & 0.027 & 0.092 & 0.092 & 0.026 & 0.105 & 0.237 \end{bmatrix}$$

The values of the gamete categories were placed in the form of a matrix, respectively:

$$g = \begin{bmatrix} 0.025 & 0.041 & 0.034 & 0.104 \\ 0.067 & 0.103 & 0.143 & 0.456 \end{bmatrix}$$

Having all the necessary data, there were compared the product values of the gametes categories frequency among which there must be equality, under genetic equilibrium conditions:

$$g_{11} \times g_{33} \times g_{17} \times g_{39} = g_{31} \times g_{13} \times g_{37} \times g_{19}$$

$$0.025 \times 0.103 \times 0.034 \times 0.456 \neq 0.067 \times 0.041 \times 0.143 \times 0.104$$

$$0.000039 \neq 0.000041$$

Since the conditions of equilibrium are not satisfied, the Great White breed sample cannot be considered in genetic equilibrium at the moment of the study.

The Hardy-Weinberg equilibrium rarely applies in reality (www.nature.com).

C. *P 2000 Synthetic Line Sample*

As regards the state of equilibrium in the three loci, the genotypic matrix was established on the basis of genotypic combinations categories weights in the LP sample:

$$G = \begin{bmatrix} 0 & 0 & 0.015 & 0 & 0 & 0.061 & 0 & 0 & 0.121 \\ 0 & 0.015 & 0 & 0 & 0.061 & 0 & 0.076 & 0.136 & 0.030 \\ 0 & 0.030 & 0.061 & 0 & 0 & 0.167 & 0 & 0 & 0.227 \end{bmatrix}$$

For the gamete matrix, there was established the following relationship:

$$g = \begin{bmatrix} 0.011 & 0.057 & 0.080 & 0.208 \\ 0.026 & 0.171 & 0.080 & 0.367 \end{bmatrix}$$

In the case we studied, the equilibrium condition:

$$g_{11} \times g_{33} \times g_{17} \times g_{39} = g_{31} \times g_{13} \times g_{37} \times g_{19}$$

isn't fulfilled, respectively:

$$0.011 \times 0.171 \times 0.080 \times 0.367 \neq 0.026 \times 0.057 \times 0.080 \times 0.208$$

$$0.000055 \neq 0.000025$$

This means that the sample analysed did not have an equilibrium genetic structure. The Hardy-Weinberg equilibrium describes an idealized state, and genetic variations in nature can be measured as changes from this equilibrium state (www.nature.com).

D. *345 Synthetic Line Sample*

In order to estimate the equilibrium state, according to the twenty-seven possible genotypic combinations among prealbumins, transferins and serum amylases, the genotypic matrix was drawn up:

$$G = \begin{bmatrix} 0 & 0 & 0 & 0 & 0.014 & 0.028 & 0 & 0.014 & 0.069 \\ 0 & 0 & 0 & 0 & 0 & 0.083 & 0.014 & 0.028 & 0.167 \\ 0.014 & 0.042 & 0 & 0 & 0.042 & 0.055 & 0.014 & 0.097 & 0.319 \end{bmatrix}$$

The gametic matrix was structured as follows:

$$g = \begin{bmatrix} 0.004 & 0.038 & 0.024 & 0.205 \\ 0.045 & 0.080 & 0.087 & 0.517 \end{bmatrix}$$

The frequencies of the gamete categories were determined according to the frequencies of the corresponding genotypic combination categories. Knowing that the equilibrium state is achieved when the gametic matrix achieves equality:

$$g_{11} \times g_{33} \times g_{17} \times g_{39} = g_{31} \times g_{13} \times g_{37} \times g_{19}$$

it can be concluded that at the time of the study the sample within LS 345 did not have a genetic equilibrium structure, since:

$$0.004 \times 0.080 \times 0.024 \times 0.517 \neq 0.045 \times 0.038 \times 0.087 \times 0.205$$
$$0.000004 \neq 0.000030$$

CONCLUSIONS

The sample belonging to the Great White breed contains the most genotypic categories (16), and in the LP sample the fewest (12). The LS sample showed 15 genotypic categories, and the Landrace sample 13 categories. The frequency of the genotype categories found in each of the four samples was different.

Common to all four studied samples is the high proportion of individuals with the $Pa^BPa^B/Tf^BTf^B/Am^BAm^B$ genotypic combination.

The highest frequency for this genotypic combination category was determined in the Landrace breed sample (39.5%).

None of the four samples contain individuals with the following genotypic combinations $Pa^APa^A/Tf^ATf^A/Am^AAm^A$ and $Pa^APa^A/Tf^ATf^A/Am^AAm^B$, as a consequence of the small share that individuals with transferin type A had in each of the studied samples.

No genetic balance for the PaTfAm aggregate genotype was identified in any investigated population. One has not identified a genetic equilibrium for the PaTfAm aggregate genotype in any of the investigated population

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

RESEARCH ON GENETIC PROGRESS OF THE ABERDEEN ANGUS BREED IN ROMANIA

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Abstract

The Herdbook is the tool for the genetic progress of Aberdeen Angus cattle, which aims to improve the performance of the breed in Romania. The specific activities of the Herdbook are performed by the Romanian Aberdeen Angus Association in accordance with the legal provisions in force and according to ICAR - International Committee for Animal Recording. The centralization, storage and evaluation of all information for Aberdeen Angus cattle in Romania enrolled in the breeding program is done only in the software "BIDAA - Informatic Database of Aberdeen Angus". The Herdbook represents a database of ascendants, contemporaries and descendants of Aberdeen Angus cattle in Romania. Logistics and permanent monitoring for the issuance of Pedigree's, creating a database with D.N.A. of breeding bulls for verifying/establishing the identity, cattle ancestry, calculating estimated breeding values, realization type classification, authorization of the bulls and other activities to achieve the goals of the Romanian Aberdeen Angus Breeding Program.

Key words*: Aberdeen Angus, Herdbook, progress genetic.*

INTRODUCTION

All the activity of the Herdbook is coordinated and specified in the Romanian Aberdeen Angus Breeding Program which includes all the necessary information for farmers and collaborators, having as main purpose the genetic progress and the improvement of the Aberdeen Angus breed in Romania. Romanian Aberdeen Angus Association is the only one accredited for the services of drawing up and maintaining the Herdbook of the Aberdeen Angus breed in Romania, accreditation no. 7/18.11.2015.

The organization chart of the Aberdeen Angus Herdbook correlates and is subject to the performance objectives of the breeding program. Any modification, copying or appropriation of the official documents of the Romanian Aberdeen Angus Association is false and is punishable according to the legislation in force.

The identification of the animals covered by this breeding program will be carried out in accordance with International Committee for Animal Recording, European and national legislation.

The Aberdeen Angus breed Herdbook in Romania recognizes the official information and documents of origin coming from other Herdbooks that respect the European legislation. Members have the obligation to send documents (annexes) every three months or to enter in the software program BIDAA (Informatic Database of Aberdeen Angus) all farm events - mounts, calving, sales and slaughtering.

Failure to comply with the rules of this improvement program has the following consequences for breeders: minutes (warnings) are drawn up if the farmer does not comply with the weighing control data in farm, does not send the notification documents on time (annexes), does not pay the invoices on time.

For the registration of an animal from another Herdbook it is necessary to present the completed documents certificate in accordance with Commission Decision 2005/379/EC and Regulation no. 1012 of June 8, 2016 of the European Parliament and of the Council of the

European Union resulting in the entry in a section of the Herdbook.

In Romania, the Aberdeen Angus breed first appeared during the years 1958-1961, breeding bulls being brought to be used in industrial crosses with poorly productive cows from local breeds in order to obtain hybrids with good results for beef production.

The first embryo transfer was made in 2000 at the Zănești farm of those from TCE 3 Brazi Piatra Neamț. Cattle from the Romanian Simmental, Holstein and Brown breeds were synchronized and the embryos were brought by Dr. Popescu Alexandru, Dr. Parchițianu Ioan Vasile and Prof. Robertson from the United States of America. These were just two uninterrupted initiatives for the Aberdeen Angus breed in Romania. During 2008, the first massive import of Aberdeen Angus cattle from Germany took place. It was a group of 120 heifers, who found their new shelter on a newly established farm near Sibiu, Transylvania. Due to fluctuations and the general decline in the price of a litre of milk, many farmers later began to opt for crosses and purchases of Aberdeen Angus cattle. Romanian Aberdeen Angus Association currently has over 1100 members from all regions of the country who own over 70.000 Aberdeen Angus cattle. Romania has recently become the country with the largest herd of Aberdeen Angus cattle in Europe (Gociman, I. et al., 2019; Vidu, L. et al., 2015).

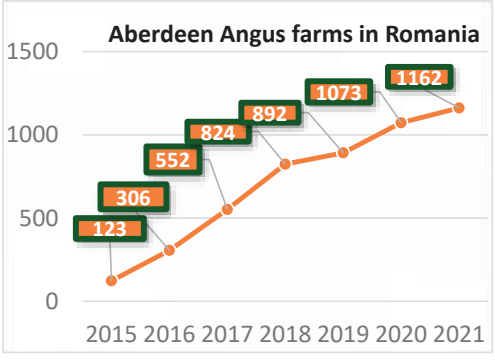

Figure 1. Evolution of Aberdeen Angus farms in the Herdbook

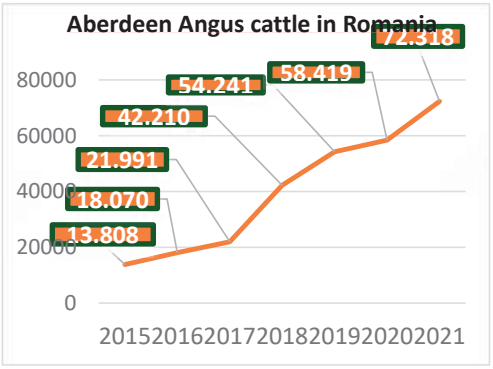

Figure 2. Evolution of Aberdeen Angus cattle in the Herdbook

MATERIALS AND METHODS

The official performance control for meat production is carried out by the control methods A, B and C. There are two mandatory controls weighing per year for calves from 90 days until 410 days old like International Committee for Animal Recording rules. All these performance weights are then sent to the Herdbook where they will be calculated reference weights, average daily gain and estimated breeding values.

With this performance we calculate for four traits like International Committee for Animal Recording rules for birth weight, weaning weight seven months, ten months and twelve months.

Directions and objectives in the improvement of the Aberdeen Angus breed in Romania

1. Maintaining reproductive precocity.
2. Development of reproductive parameters such as: fecundity, fertility, vitality and viability.
3. Low specific consumption for achieving large average daily gain increases (ADG).
4. Obtaining cattle that ensure a superior quality of the carcass.

Table 1. Objectives of the Romanian Aberdeen Angus Breeding Program

Character	Desired type in the future	Current herd performance	Improvement Objectives
Birth weight	25 kg	30 kg	Decreased birth weight
Weaning weight of breeding bulls	230-260 kg to 200 days	200-220 kg to 200 days	Improving the weight of bulls
Calf weaning weight	200-220 kg to 200 days	165-185 kg to 200 days	Improving the weaning weight of breeding calves
Weight at 10 months	340-360 kg to 300 days	290-310 kg to 300 days	Improving the weight of bulls
Weight at 12 months	430-460 kg to 365 days	380-400 kg to 365 days	Improving the weight of breeding calves

The objectives of improving the number of Aberdeen Angus cattle in Romania according to the market and farmers requirements, current and future are defined (Table 1). For these characters, improvement values will be calculated with the B.L.U.P. method.
Before the official performance control for meat production in the Aberdeen Angus breed begins, the origin of each animal must be certified (validated) and entered only in the software program BIDAA, granted by the Romanian Aberdeen Angus Association for calculation and estimation of performance for the genetic value of an animal (estimation method that contains the relation between the cattle population traits and the performance traits of each one – animal model Henderson - B.L.U.P.). The identification and individualization are attributed to the breeders to ensure the notification documents.

Total selection index:

EBV Total = 0% * EBV birth + 40% * EBV weaning-200 days + 30% * EBV 300 days+ 30% * EBV 365 days

Following the genetic evaluation, the cattle will be chosen for breeding in descending order based on the EBV total according to the needs of each farmer. For each character, improvement values will be calculated by the BLUP method.
In order to define a broader framework for genetic evaluation of animals, compared to the selection index (BLP) method, Henderson (1949, 1963 and 1973) developed a statistical methodology for the simultaneous evaluation of the fixed and random effects of the mixed model, called BLUP - Best Linear Unbiased Predictors (method of the best unbiased linear predictors).
The Herdbook of the Aberdeen Angus breed is divided as follows:
- Youth class
a) Subclass of calves:
- all males up to the age of seven months, coming from parents registered in the main section and who are 100% purebred Aberdeen Angus, will be enrolled in the youth class;
- all females up to the age of seven months, from parents registered in the main section and who are 100% purebred Aberdeen Angus;
- all females up to the age of seven months (at least 93.75% F4 Aberdeen Angus) from the additional section mothers (minimum 87.5% F3 Aberdeen Angus) and the father from the main section 100% Aberdeen Angus.

b) Subclass A:
- male and female youth originating over two generations with parents and grandparents enrolled in the main section;
- ADG - average daily gain increase over 700 g females; 900 g males at any weighing;
- percentage of 100% Aberdeen Angus blood;
- minimum age 7 months.

c) Subclass B:
- male and female youth originating over two generations with parents and grandparents enrolled in the main section, Aberdeen Angus 100% blood percentage;
- female youth of two generations originating from a purebred father and a mother of the following ancestry; the mother and maternal grandmother registered in the supplementary section and the father and the two grandparents registered in the main section. Aberdeen Angus blood percentage minimum 93.75% (F4);
- male and female youth who do not meet the performance requirements for admission to the youth class - subclass A;
- minimum age 7 months.

- Bull class
a) Subclass A:
- they come from the main section of the youth class subclass A, originate for two generations and are 100% Aberdeen Angus;

- ADG - average daily gain increase of at least 900 g. for recorded weighings;
- DNA or any other test based on DNA genome analysis;
- have estimated breeding value;
- type classification - minimum score, average 7 in each category.

b) Subclass B:
- they come from the main youth class section, originate for two generations with parents and grandparents in the main section and are 100% Aberdeen Angus;
- males that do not meet the admission performances in the bull class - subclass A;
- DNA or any other test based on DNA genome analysis;
- have estimated breeding value.

- Cow class
a) Subclass A:
- they come from the main section youth class subclass A, originate over two generations and be 100% Aberdeen Angus;
- ADG - average daily gain increase over 700 g at any weighing;
- have estimated breeding value;
- type classification - minimum score, average 6 in each category.

b) Subclass B:
- they come from the main section youth class subclass B, originate over two generations and be 93.75% Aberdeen Angus, the father to come from the main section;
- females that do not meet the admission performances in the cow class - subclass A;
- have estimated breeding value.

Additional youth class - commercial
- crossbreed young females to be obtained by crossbreeding with the Aberdeen Angus breed (young female F2 75% Aberdeen Angus and F3 87.5% Aberdeen Angus). Must have documents certifying the origin of the Aberdeen Angus breed;
- females until the first calving;
- ADG average daily gain increase over 500 g at any weighing;
- have the *akeratos* character specific to the Aberdeen Angus breed.

Additional cows' class - commercial
- crossbreed females with Aberdeen Angus after the first calving (females F1 50% Aberdeen Angus after a first calving);
- mixed-breed females obtained by absorption crossing which are the subject of the Herdbook (females F2 75% Aberdeen Angus and F3 87.5% Aberdeen Angus);
- ADG average daily gain increase over 500 g. at any weighing;
- have the *akeratos* character specific to the Aberdeen Angus breed;
- they have registered at least one calving.
In order to receive the pedigree, the Aberdeen Angus cattle in Romania must respect the following aspects:

For females:
- Minimum age - 12 months;
- The origin is known for two generations;
- The birth weight is known;
- The race percentage should be at least 93.75% Aberdeen Angus (F4).
- It is known at least one weight for a reference age, and the average daily gain increase is at least 600 g/day;
- They come from a father authorized for natural mounting/artificial insemination and DNA testing;
- Females older than 32 months must have registered a calf;
- The name is known (farm name + mother's name + letter assigned internationally to the year of birth + last 3 digits of the ear tag).

For males:
- Minimum age 12 months;
- The origin is known for two generations;
- The birth weight is known;
- The race percentage must be 100% Aberdeen Angus;
- It is known at least the weight at 200 days and/or 365 days, and the average daily gain increase is at least 900 g/day;
- They have DNA profiles;
- The name is known (farm name + father's name + letter assigned internationally to the year of birth + last 3 digits of the ear tag).
- Letters allocated internationally at the year of birth: for 2015 - letter R, year 2016 - letter S, year 2017 - letter T, year 2018 - letter U, year 2019 - letter V, year 2020 - letter W, year 2021

- letter X, year 2022 - letter Y, year 2023 - letter Z.

Type classification is made for the next traits:
1. Chest development;
2. Width of the forelimbs;
3. The aplomb of the forelimbs;
4. The aplomb of the hind limbs;
5. The size of the front train compared to the rear one;
6. Horizontal line withers - croup;
7. Clamping and circumference of the scrotum;
8. Head development;
9. Functional skills (locomotion).
Arithmetic mean total/final grade
Observations: For each character a score from 1 to 9 is given.

Measure:
Scrotal circumference (cm)
Height at withers (cm)
Height at croup (cm)
Chest depth (cm)
Hail length-tail grip (cm)
Weight (kg)
Spots/Horns
Temperament

Within the Romanian Aberdeen Angus Association, the reproduction bulls are authorized only if they have a pedigree, respect the characters of the Aberdeen Angus breed, are healthy, have type classification, have a DNA profile of maternal and paternal origin (Grosu and Gociman, 2018).
In order to be admitted for breeding, Aberdeen Angus cattle in Romania must comply with the following parameters:

Table 2. Reproduction parameters

Reproduction parameter	Number of days
Minimum gestation time	263
Maximum gestation time	297
Minimum number of days between two consecutive calvings (calving interval)	305
Minimum difference between the age of the mother and the calf	720
The minimum difference between the age of the father and the calf	720
Minimum sexual cycle	18
Maximum sexual cycle	28

If the reproductive parameters are not met, DNA tests must be performed confirming maternal and/or paternal origin. D.N.A. profile and parentage are made with the Microsatellite method with authorized laboratory.

RESULTS AND DISCUSSIONS

With all these objectives and rules of the Romanian Aberdeen Angus Breeding Program in the following tables you will find the genetic progress for the four quantitative traits: birth, weaning 200 days, 300 days and 365 days.
In the following graphic (Figure 3) you can find the evolution of the birth weight for the Aberdeen Angus youth cattle in 2018, 2019 and 2020. The average birth weight was 30.8 kg in 2018, 30.9 kg in 2019 and 30.7 kg in 2020.
For Aberdeen Angus breed in Romania, we want high calving ease as possible with calves that are easily fattened having subsequently a significant average daily gain.

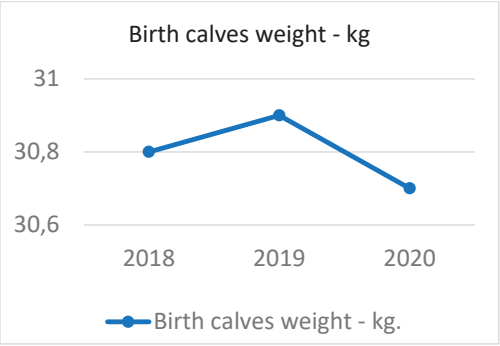

Figure 3. Evolution of birth calves' weight

In the following graphic (Figure 4) is the evolution of weights at seven months, ten months and twelve months for the Aberdeen Angus youth cattle in 2018, 2019 and 2020. The average weight at seven months was 206 kg in 2018, 206 kg in 2019 and 216 kg in 2020. Until this age, the calves are with their mothers, weaning taking place after seven months. The average weight at ten months was 281 kg in 2018, 285 kg in 2019 and 292 kg in 2020. The average weight at twelve months was 321 kg in 2018, 320 kg in 2019 and 383 kg in 2020.

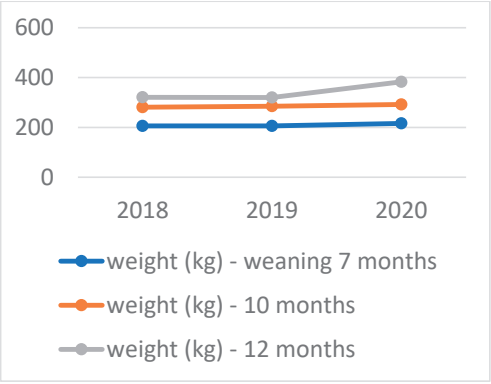

Figure 4. Evolution of weights

In the following graphic (Figure 5) is the evolution of average daily gain ADG at seven months, ten months and twelve months for the Aberdeen Angus youth cattle in 2018, 2019 and 2020. The average daily gain at seven months was 884 g in 2018, 879 g in 2019 and 927 g in 2020. The average daily gain at ten months was 833 g in 2018, 845 g in 2019 and 873 g in 2020. The average daily gain at twelve months was 795 g in 2018, 794 g in 2019 and 960 g in 2020. From year to year is an increase in the number of Aberdeen Angus reactive cattle, they better withstand the stress of weaning and this is seen in the evolution of performance.

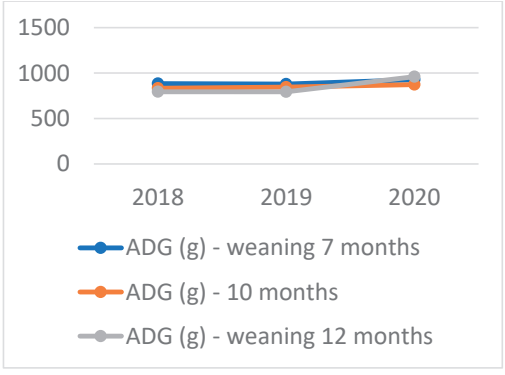

Figure 5. Evolution of average daily gain ADG

As can be seen in the graphs above, the Aberdeen Angus breed in Romania had a performance improvement every year for the four characters weight and/or average daily gain at birth, seven months, ten months and twelve months. Both, weights and average daily gain is influenced by internal factors such as genetics, physiological, species, race, age, sex and external factors such as environment, feed, water, breeding season, calving season and technology (Gociman et al., 2020).

CONCLUSIONS

Romanian Aberdeen Angus Association has complied with the Romanian Aberdeen Angus Breeding Program with annual genetical performance progress.

The large areas of natural meadows, the climate, the relief, the variation of precipitations, the soil, the quality of the fodder, are some of the great strengths of Romania to increase such a quality breed in an extensive system.

Romania has to become a European beef brand country.

ACKNOWLEDGEMENTS

This research work was carried out with the support of the Romanian Aberdeen Angus Association and the Faculty of Engineering and Management of Animal Production, University of Agronomic Sciences and Veterinary Medicine of Bucharest.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

ULTRASOUND MEASUREMENTS OF EYE MUSCLE PROPERTIES AND BACKFAT THICKNESS IN LAMBS FROM DIFFERENT GENOTYPES FED WITH DIFFERENT DIETS

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Abstract

The present study was conducted to determine eye muscle (M. Longissimus dorsi) properties of lambs from two genotypes. The depth, eye muscle area and eye muscle perimeter of Longissimus dorsi and thickness of fat covering this muscle using ultrasonic scan was determined using ultrasonic measurements from 118 lambs taken from the two different genotypes - 80 heads from Tsigai - rusty variety (40 female and 40 male) and 38 heads (30 female and 8 male) from Suffolk (50%) x German Blackface (37.5%) x Tsigai (12.5%).The animals were fed with different diets. Birth weight was significantly ($p<0.05$) affected by breed and sex. Likewise, weaning weight and weight at 5 months was found to be significantly affected by sex and breed. Mean for ultrasound measurements of eye muscle properties were highly significant ($p<0.001$; $p<0.01$) for eye muscle depth at 12 rib, eye muscle area at 12 rib and 3-4 lumbar vertebrae, and for backfat thickness in the two points; significant differences ($p<0.05$) were found for eye muscle depth and area at 3-4 lumbar vertebra, but no significant differences were found for eye muscle perimeter ($p>0.05$). The lamb sex and breed was found to be significant variable for eye muscle area and the lamb diet for eye depth at 3-4 lumbar vertebrae. Regarding the phenotypic correlations between ultrasound measurements with weight at 5 months, from a total of 45 traits couples, 46.66% are small correlation (0.00-0.30), 26.67% are medium to high correlations (0.31-0.60) and high correlations recorded 26.67% (0.61-1.00). Due to the lack of labor in agriculture, in the last years many sheep breeders do not want to milk the sheep, they prefer to let the lambs graze along with their mothers, so that the lambs are marketed at the age of 4-5 months. It is extremely difficult to take measurements on carcasses in these regions as lambs are mainly marketed or slaughtered as small groups or individually and abattoirs do not record any measurements on carcasses characteristics. In this situation, information on body composition of lambs can be obtained practically by ultrasonic measurements on live animals. When combined in a breeding program with lamb weight at 5 months or market weights, these measurements will provide a way to increase both meat yield and the quality of lambs.

Key words*: genotype, lamb, Longissimus dorsi, Tsigai, ultrasound.*

INTRODUCTION

Real-time ultrasonography is a non-invasive technology that allows carcass quality to be assessed without damaging the product (Delfa et al., 1996; Fernández et al., 1998; Mendizabal et al., 2003). The relatively low cost and ease of portability of ultrasound equipment has led ultrasound measurements to be incorporated into national genetic programs for lamb carcass quality improvement in many parts of the world (Stanford et al., 1998).

The ultrasound measurement is a new technique which is a non-invasive, efficient grading method for classification and quantification of the animal carcasses in their early life as well as the further usage of animals for reproduction. The measured parameters (subcutaneous fat layer thickness, muscle eye area and muscle depth) add new selection indices (muscle depth and subcutaneous fat layer thickness) to the classical ones (body weight, carcass meat). The ultrasound method is mostly used for sheep carcass grading in the western EU countries which have a long tradition in sheep rearing and breeding for meat production. Applications of this modern technology are already deployed in the UK,

New Zealand and Ireland for the breeding programmes (Fogarty et al., 1992, 1995; Wilson, 1992; Russel, 1995; Larsgard & Kolstad, 2003), showing very good correlations with the classical method. Fernández et al. (1997) stated close correlations between the ultrasound measurements and the carcass measurements of LD muscle for muscle eye area, muscle depth and thickness of the subcutaneous fat layer (0.88, 0.74 and 0.56, respectively) in Manchego lambs. Moderate correlations between the ultrasound measurements and the carcass assessment for muscle eye area and weaning body weight, and between the thickness of the subcutaneous fat layer and weaning body weight were determined by Fernández et al. (1998) and Ibrahim et al. (2007). Emenheiser et al. (2010) showed the necessity of validating the ultrasound method utilization to determine lamb carcass composition for meat production and showed the advantages of this method. Therefore, ultrasound can offer breeders, producers and researchers the ability to estimate carcass composition traits in vivo and thus contribute knowledge to precision of breeding, management and marketing decisions (Leeds et al., 2008).

The study was conducted to determine backfat thickness and Longissimus dorsi muscle properties of lambs from Tsigai breed and crossbred Suffolk (50%) x German Blackface (37.5%) x Tsigai (12.5%) lambs using ultrasonic measurements and to evaluate the effects of diet, genotype, and sex on these parameters. By providing the first reference for these phenotypic parameters with ultrasonic measurements, this study may assist with the initiation of a genetic breeding program which includes ultrasonic measurements to improve meat yield and in turn the quality of lambs from Tsigai - rusty variety.

MATERIALS AND METHODS

The present research was conducted in Experimental Base Reghin of Research Institute for Sheep and Goat Palas Constanta, Mures County, 46°46' N/ 22°42'E; 395 m altitude; annual rain fall varies between 650-700 mm; average temperatures 19/–3°C during summer/winter).

Animal management

118 lambs (one hundred and eighteen) separated into five lots, from Tsigai - rusty variety (80 heads, from which: 40 males - lot 1 (L1) and 2 (L2) - 20 heads/lot; 40 females - lot 3 (L3) and crossbred lambs Suffolk (50%) x German Blackface x Tsigai (SxBFxTi) - 30 female - lot 4 (L4) and 8 males - lot 5 (L5) was used as animal material in this study. The sheep mothers were raised in extensive conditions, were kept on pastures throughout the year. However, concentrate are used in feeding of ewes as supplement during some critical periods, such as late gestation and 2 month after lambing. Lambs were born from January through March. At birth or shortly thereafter, lambs were identified with ear tags and weighed (± 0.1 kg). Sex, date of birth, type of birth, dam and ram group were recorded. The lambs were also weighed monthly (± 0.1 kg) up to 5 months age. Ewes and their lambs were kept together under the same management condition for two months after lambing. Lambs were weaned at approximately 58-62 days to Tsigai breed and 57-62 days of age to crossbred lambs. In the suckling period, the diet was formulated for 300 g/head/day growth potential according to NRC (1985) requirements (135 g DP and 10.89 MJ NE).

The structure of concentrated fodder was: 30% corn flour, 30% barley flour, 25% corn grain, 11.25%, sunflower groats, 2.25% calcium and 1.5% salt. After weaning up to 5 months, the five lots of lambs were fed with different diet, the lambs were raised on shelter, and the diet was offered *ad libitum.* For the lot 1, the structure of concentrate fodder was the same like in the suckling period (from birth up to end of fattening), for the lot 2 it was different in comparison to suckling period and separated into two phases during the fattening period, and for the lots 3, 4, and 5 the diet was without differences between the 3 lots during the fattening period. The structure of concentrate fodder is presented in Table 1. Additional, for all lots, in the ration was added hill hay.

The lambs were measured for birth weight (BW), weaning weight (WW), and weight at 5 months (W5M), musculus Longissimus dorsi (LD) depth (LMD), area (LMA) and perimeter (LMP) and backfat thickness (BF) covering this muscle at the area between the 12th and 13th

rib, and between 3 and 4 lumbar vertebrae using ultrasonic scan.

Table 1. Structure of concentrate fodder used in fattening experiment with lambs from different genotyp

Characteristics	After weaning					
	L1	L2		L3	L4	L5
	6 April 15 July	6 April 6 Juni	7 Juni 15 July	6 April 15 July	6 April 15 July	6 April 15 July
Corn flour (%)	30.0	4.00	20.0	4.00	4.00	4.00
Characteristics	After weaning					
	L1	L2		L3	L4	L5
	6 April 15 July	6 April 6 Juni	7 Juni 15 July	6 April 15 July	6 April 15 July	6 April 15 July
Barley flour (%)	30.0	4.00	20.0	4.00	4.00	4.00
Corn grain (%)	25.0	90.00	50.0	90.00	90.00	90.00
Sunflower groats (%)	11.25	1.50	7.5	1.50	1.50	1.50
Calcium (%)	2.25	0.30	1.5	0.30	0.30	0.30
Salt (%)	1.5	0.2	1.0	0.2	0.2	0.2
Dry matter/ kg concentrated fodder	820	830	830	830	830	830
Digestible protein g/kg dry matter	135	100	118	100	100	100
NE MJ/kg dry matter	10.89	11.29	10.89	11.29	11.29	11.29

Wool was removed from measurement areas by shearing before ultrasonic scanning. The ultrasonic measurements were performed by an experienced operator *in vivo* using a HS-1600 (HONDA ELECTRONICS CO.) ultrasonic machine with a HLV 7218 linear probe and the following operating frequencies: 1.5/2.0/3.0 Mhz.

All recorded images were then analysed with ImagesJ program.

After scan image capture, the muscle depth, Longissimus dorsi area and perimeter, and the thickness of backfat at the two points was measured using the electronic calliper of the scanner. The resolution of scanner calliper was 0.01 cm. Longissimus dorsi area and perimeter was measured on the same image after the borders of muscle had been drawn.

Statistical data processing was performed with the ANOVA program, and the tests used were the "Tukey" tests.

RESULTS

The body evolution, total gain and average daily gain (ADG) from birth to end of fattening are presented in Table 2.

The minimum average live weight of lambs at birth was comprised between 4.22 kg at L3 (female from Tsigai breed) and 4.95 kg at L5 (male from crossbred lambs). Significant differences ($p<0.05$) were recorded between L3 and the lots L4 and L5 with regard at birth weight. The analyses have indicated significant effects on weight at 5 months of breed and sex. Male weights of female lambs in L4 were found 7.12 kg heavier than female lambs in L3. Also, the weight at 5 months of female from L4 were found 1.65 kg heavier than male lambs in L2. Male lambs in L5 were found to be significantly heavier than the females from the same genotype (48.57 vs. 41.83 kg; $p<0.01$). At the same time, significant differences were found between the lots of lambs from the two genotypes, so, between L5 and L1 ($p<0.05$) and between L5 and L2 ($p<0.001$). Significant differences ($p<0.001$) were found between all lots of lambs and lambs of female from Tsigai sheep (L3). No significant differences were found between the lots of male lambs from Tsigai sheep, therefore the weight at 5 months was not influenced by the diet.

The statistics for ultrasonic measurements of the eye muscle properties and lambs weights of lambs at 5 month are given in Table 3. The difference observed due to the sex of lambs was significant for backfat thickness ($p<0.05$), muscle depth ($p<0.01$) and eye muscle area ($p<0.001$). Between the means of lots were highly significant differences ($p<0.001$) for eye muscle area at the lots L2 (male) and L4 (female), and significant ($p<0.01$) for the lots of female (L3, L4), compared to the lots L1 and L2. The eye muscle area was higher to female lambs from the two genotypes, compared to male lambs from Tsigai breed (1.59 cm^2 higher L4 compared to L1 and 1.97 cm^2 L4 vs. L2, respectively; likewise, 1.08 cm^2 higher to L3 compared to L1 and 1.46 cm^2 higher L3 compared to L2, respectively). The difference observed due to the breed of lambs was significant for backfat thickness ($p<0.01$) and eye muscle area ($p<0.01$, $p<0.001$). From the Table 3 it can be observed, that the lots of crossbred lambs have the lowest backfat thickness, even if those have the highest body weight. At the same time, the lots of crossbred have the highest eye muscle area.

The diet of lambs were significant sources of variation ($p<0.05$) for the ultrasonic measurements for LD muscle depth between 3-4 lumbar vertebra and eye muscle area between 3-4 lumbar vertebra.

Table 2. Mean (± SE) for body evolution, total gain and ADG of lambs from birth to end of fattening

Specification	Genotyp				
	L1 Tsigai (M) n = 20	L2 Tsigai (M) n = 20	L3 Tsigai (F) n = 40	L4 SxBFxTi (F) n = 30	L5 SxBFxTi (M) n = 8
BW, kg	4.44 ± 0.16	4.44 ± 0.15	4.28 ± 0.10^{a}	4.62 ± 0.12^{b}	4.95 ± 0.23^{b}
WW, kg	20.16 ± 0.48^{aA}	20.17 ± 0.51^{aA}	18.22 ± 0.48^{bAD}	23.01 ± 0.56^{B}	24.90 ± 1.08^{C}
W5M, kg	42.23 ± 1.33^{aA}	40.18 ± 1.33^{A}	34.71 ± 0.94^{C}	41.83 ± 1.08^{A}	48.57 ± 2.10^{bB}
Total gain birth-weaning, kg	15.72 ± 0.69 ^{aA}	15.73 ± 0.69 ^{aA}	13.95 ± 0.49^{bA}	18.38 ± 0.56^{bB}	19.95 ± 1.09^{B}
Total gain weaning - end of fattening, kg	22.07± 0.98^{A}	20.01± 0.98^{A}	16.49 ± 0.69^{aB}	14.20 ± 0.80 ^{bB}	18.72 ± 1.55^{C}
Total gain birth-end of fattening, kg	37.79 ± 1.02^{aA}	35.74 ± 1.38^{A}	30.44 ± 0.93^{C}	37.21 ± 1.08^{A}	43.62 ± 2.09^{bB}
ADG birth-weaning, kg	238.10 ± 11.16^{a}	277.10 ± 11.16^{bA}	235.85 ± 7.89^{B}	306.11 ± 9.11^{acA}	334.07 ± 17.65^{C}
ADG weaning - end of fattening, kg	220.70 ± 9.37^{A}	200.05 ± 9.37^{A}	169.36 ± 6.63^{B}	137.86 ± 7.65^{AC}	187.77 ± 14.81^{D}
ADG birth-end of fattening, kg	225.83 ± 7.46^{A}	225.33 ± 7.46^{A}	193.72 ± 5.28^{B}	227.71 ± 6.09^{AC}	273.67 ± 11.80^{D}
Age at weaning, days	66.35 ± 2.06^{aA}	57.90 ± 1.82^{B}	59.63 ± 1.29^{B}	60.60 ± 1.49^{b}	58.89 ± 2.88^{b}
Age at end of fattening, days	167.35 ± 1.45^{A}	158.90 ± 2.06^{B}	156.80 ± 1.45^{BC}	163.60 ±1.68^{ABD}	157.13 ±3.25^{BCD}

Means with different superscripts ($^{a, b, c, d}$) in each traits differ (P< 0.05).
Means with different superscripts ($^{A, B, C, D}$) in each traits differ (P< 0.01 and P< 0.001).

Table 3. Mean (± SE) for ultrasound measurements of eye muscle properties and weights of lambs at end of fattening

Specification	Lot 1 n = 20 male	Lot 2 n = 20 male	Lot 3 n = 40 female	Lot 4 n = 30 female	Lot 5 n = 8 male
Fat depth on 12 rib (mm)	6.46 ± 0.25	6.96 ± 0.25^{A}	6.46 ± 0.18	6.15 ± 0.21^{B}	6.36 ± 0.40
Fat depth between 3-4 lumbar vertebra (mm)	6.44 ± 0.24^{A}	6.55 ± 0.24^{A}	6.01 ± 0.17^{A}	5.35 ± 0.19^{B}	6.12 ± 0.37
LD Muscle depth on 12 rib (mm)	23.36 ± 0.68	21.66 ± 0.68^{A}	23.27 ± 0.48	24.05 ± 0.56^{B}	24.11 ± 1.08
LD Muscle depth between 3-4 lumbar vertebra(mm)	24.24 ± 0.62^{a}	22.33 ± 0.62^{b}	22.52 ± 0.44^{b}	22.67 ± 0.51	23.28 ± 0.99
Eye muscle area on 12 rib (cm^2)	14.25 ± 0.45^{A}	13.87 ± 0.45^{A}	15.33 ± 0.32^{B}	15.84 ± 0.37^{B}	16.30 ± 0.72^{B}
Eye muscle area between 3-4 lumbar vertebra (cm^2)	13.78 ± 0.46^{a}	12.31 ± 0.46^{Ab}	13.69 ± 0.33^{ac}	14.11 ± 0.38^{B}	15.15 ± 0.73^{B}
Eye muscle perimeter on 12 rib (mm)	167.00 ± 3.16	174.25 ± 3.16	171.73 ± 2.23	174.90 ± 2.58	177.13 ± 4.99
Eye muscle perimeter between 3-4 lumbar vertebra (mm)	164.40 ± 3.12	162.10 ± 3.12	164.43 ± 2.20	167.43 ± 2.54	170.13 ± 4.93
Live weight (kg)	42.23 ± 1.33^{A}	40.18 ± 1.33 ^{A}	34.71 ± 0.94^{C}	41.83 ± 1.08^{A}	48.57 ± 2.10 ^{B}

Means with different superscripts ($^{a, b, c, d}$) in each traits differ (P<0.05).
Means with different superscripts ($^{A, B, C, D}$) in each traits differ (P<0.01 and P<0.001).

Regarding the phenotypic correlations between ultrasound measurements with weight at 5 months, from a total of 45 traits couples 46.66% are small correlation (0.00-0.30), 26.67% are medium to high correlations (0.31-0.60) and high correlations recorded 26.67% (0.61-1.00). The correlation between backfat thickness and Longissimus dorsi depth, area and perimeter were found to be negative. All others correlation were found to be positive. The correlation between Longissimus dorsi area with perimeter were highest (0.75 at 3-4 lumbar vertebrae and 0.70 at 12 rib, respectively) (Table 4). There were moderate correlations between the weight of lamb at 5 months and the measurements of Longissimus dorsi depth and area, but the correlation of weights at 5 months with backfat thickness and perimeter was lower.

Significant ($p<0.001$) differences were found between backfat thickness at 3-4 lumbar vertebrae and at 12 rib, as well between Longissimus dorsi area with depth, perimeter and body weight at 5 months.

Table 4. Correlation coefficients between ultrasound measurements and weight at 5 months (n = 118)

Traits	BFT12	BFT34	LMD12	LMD34	LMA12	LMA34	LMP12	LMP34	W5M
BFT12	1.00								
BFT34	0.43***	1.00							
LMD12	-0.02	-0.01	1.00						
LMD34	0.26	0.01	0.29	1.00					
LMA12	0.01	-0.04	0.55***	0.34***	1.00				
LMA34	0.01	0.02	0.44***	0.54***	0.70***	1.00			
LMP12	0.16	0.16	0.09	0.22	0.70***	0.42***	1.00		
LMP34	-0.01	0.10	0.26	0.20	0.51***	0.75***	0.45***	1.00	
W5M	0.21	0.08	0.36***	0.36***	0.40***	0.37***	0.27	0.20	1.00

BFT12, BFT34: BFT: Backfat Thickness; LMD12, LMD34: Longissimus Muscle Depth; LMA12, LMA34 - Longissimus Muscle Area; LMP12, LMP34 - Longissimus Muscle Perimeter; W5M - Weight at 5 Months; ***$p<0.001$

In this study, average lamb weight was comprised between 34.71 kg at L3 (the lot of female from Tsigai sheep) and 48.57 kg at L5 (male from crossbreed) and the average age ranged from 156 to 167 days, depending on the lot. Animal age at the time of measurement is important, as variation may exist between genetic evaluation programs which are based on ultrasonic measurements, if these scan measurements are ascertained at different time periods. Australia's genetic evaluation and performance testing program, LAMBPLAN, allows ultrasonic measurement for lambs to be taken over a wide range of ages, 5 to 18 months (Gilmour et al., 1994). Others, such as Suffolk sire-reference schemes in Canada (Gallivan & Hosford, 1997) and Britannia (MLC, 1987) target measurement at 100 and 147 days of age, respectively.

With respect to lamb weight, environmental, and genetic factors, the results observed here show differences between lots, depending on genotype and administered diet. However, the effect of diet had showed significant differences ($p<0.05$) between lots from Tsigai lambs (L1 and L2) with regard at LD muscle depth between 3-4 lumbar vertebra and eye muscle area between 3-4 lumbar vertebra. With respect at genotype, the value of crossbreed lambs appear superior with regard at body weight at birth, weaning and end of fattening, compared to the lots from Tsigai lambs. Daily gain was higher at crossbreed, and as expected, male lambs were higher performing than their female counterparts, in both genotypes (Tsigai and crossbreed lambs). The effects of genotype, sex and diet were observed in this experiment.

For the five lots of lambs that were assessed, the average backfat thickness, LD depth, LD eye muscle area and perimeter measured by ultrasound. The average backfat thickness in the two points were slower at lots of crossbreed (female and male), followed by the lot of females from Tsigai sheep, although the crossbreed registered the biggest live weight. The area of the eye muscle at 12 rib had the

highest values at L5 (16.30 cm^2) followed of L4 (15.84 cm^2) and L3 (15.33 cm^2). Approximately the same tendency is maintained in the 3-4 lumbar vertebra, with the mention that here, the L1 has higher values than the L3. From the data of table 3, it is observed that the groups of females have higher values of the eye muscle area, compared to the groups of males of crossbreed (L5).

The area of the eye muscle was recorded as, 8.95, 9.67 and 10.85 cm^2 for Manchego, Merinos and Ile de France x Merino lambs, respectively, with a live weight ranging from 22 to 28 kg (Fernandez et al., 1997). The fat thickness for these genotypes was also recorded as 3.28, 3.83 and 4.10 mm, respectively. Similarly, Stanford et al. (2001) assayed 90 day old male and female lambs with live weights of 27.3 and 25.3 kg. The loin eye area was recorded as 7.15 and 7.42 cm^2 and the fat thickness of these sheep were recorded as 2.74 and 2.96 mm, respectively.

The correlation coefficients obtained in the present study are in the range previously reported in literature (Fernandez et al., 1997; Conington et al., 2001; Safari et al., 2005). Fernandez et al. (1997) stated the correlation coefficient of muscle area with muscle depth was reported as 0.56. Findings in present study were in agreement with their values. Lazar et al. (2016) had found very close correlations at Tsigai Blackhead of Teleorman between the weight at the age of 2.5 months and subcutaneous fat layer thickness, muscle depth and muscle eye area (0.72, 0.71, and 0.82, respectively). Similar results have been reported in Kivircik lambs by Ibrahim et al. (2007), who found strong correlations between body weight at birth and muscle depth (0.609) and muscle eye area (0.649). The same authors also reported strong correlations between the muscle eye area and muscle depth (0.845).

Ultrasonic measurement technology has been used in selection programs to improve growth and carcass traits in sheep (Simm et al., 1987; Larsgard & Kolstad, 2003). The advantage of this method is that it can be used on live animals at relatively low costs (Conington et al., 1995; Larsgard & Kolstad, 2003). In addition, heritability estimates for ultrasonic fat and muscle measurements were moderate to high (Fogarty, 1995; Jones et al., 2004; Safari et al., 2005). The results reported here with Tsigai and crossbreed lambs will be useful for future studies including genetic improvement of meat quality in lambs. In many parts of the world (UK, Australia, New Zealand, Denmark, Finland, Norway) ultrasound measurements are incorporated into national genetic evaluation programs or into selection indices to achieve high quality lamb carcasses (Stanford et al., 1998).

CONCLUSIONS

Lambs are being sent to market at weaning (70-90 days), or the lambs graze along with their mothers, so that the lambs are marketed at the age of 4-5 months. It is very difficult to take individual carcass measurements as lambs are generally marketed or slaughtered in small groups or individually in an unplanned manner. Ultrasonic scans on live animals may be used in breeding programs for lambs from Tsigai breed - rusty variety, along with weaning or market weight, to increase meat yield and quality of lambs. The rate of genetic improvement for growth and carcass characteristics of lambs can be accelerated by a breeding program including ultrasonic measurements along with other records, such as live weight or live weight gains in some periods.

ACKNOWLEDGEMENTS

This research work was carried out with the support of Ministry of Agriculture and Rural Development of Romania, Department of Research and also was financed from Project ADER 8.1.1/2019.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

COMPARATIVE CHARACTERISTICS OF EXTERIOR AND ECONOMICALLY USEFUL FEATURES OF DAUGHTERS OF DIFFERENT BULLS

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Abstract

There are presented the results of studies of the exterior and economically useful characteristics of first calf heifers of the local generation of the Holstein breed, bred in a herd of Joint-Stock Company "Aydyn", Komrat, Administrative and Territorial Unit Gagauzia, Republic of Moldova. The bone index in comparison with the standard turned out to be on average 8.3% lower, which is due to the high height at the withers of Holstein cows. Comparative analysis of milk productivity of daughters of various bulls showed that milk yield for 305 days of lactation of daughters of bull Forms 999 is 155 kg more milk than that of daughters of bull Kiperush 79. Realization of the genetic potential of first-calf heifers of local selection was at the level of 91.4-92.1% of the productivity of full-aged cows, with a fat content of 3.82-3.89%. The relationship between the milk yield and the percentage of fat in the milk of the daughters of the analyzed bulls was in a positive correlation from the weak (+0.034, daughter of Kiperush 79), to moderate (+0.369, daughter of Forms 999).

Key words*: body builds indices, correlation, exterior, first-calf heifer, milk yield.*

INTRODUCTION

Cattle breeding are one of the leading industries in Animal Science, which determines the wide distribution of cattle in various natural and economic zones of the world. The exterior or the appearance of an animal is formed under the influence of the genotype and living conditions of the organism. The combined action of these two factors is carried out in the process of individual development of animals.

Examination of the exterior allows determining the relationship that exists between the appearance of the animal and its productivity. The correct physique and strong constitution may indicate the resistance of animals to adverse external influences, the ability to long-term economic use.

When breeding for the milk production of cows, individual populations of cattle, evaluation of the exterior of animals is of no small importance, the features of which have a certain connection with the duration of the productive use of highly productive cows (Ovchinnikova et al., 2016; Babich et al., 2018; Kostomakhin et al., 2011).

As the exterior is closely related to milk productivity, in the selection of the exterior on animals, takes place an indirect selection and productivity. Exterior assessment includes as the general appearance of the animal (typicality, manifest of dairy forms, presence of defects and deficiencies) as specific measurements of individual parts of the animal's body, anatomically related to each other.

For the last 10 years, Holstein cattle have been imported to the Republic of Moldova from such European countries as Holland, Germany, France, Austria and Hungary. These animals are distinguished by increased productivity, good health and are able to acclimatize and adapt to the conditions of various climatic zones of the republic.

Research has been carried out and studied the milk productivity (first – fourth lactation) and the exterior of Holstein cows of Dutch and German breeding for the first and third lactations in the herd of Joint-Stock Company

"Aydyn", (Foksha et al., 2016; Foksha et al., 2019). It has been established that under the new housing conditions, Dutch and German Holstein cows realize their genetic potential at a high level (Konstandoglo et al., 2018; Foksha et al., 2018), which is facilitated by the appropriate conditions of housing and feeding of animals. It should be noted that the Holstein cattle, purchased from Holland and Germany, successfully adapted to the conditions of the south of the Republic of Moldova, in particular in herds of Joint-Stock Company "Aydyn" and Society of limited liability "Doksancom".

The purpose of our research is to study the exterior and economically useful characteristics of first-calf cows of the local generation of the Holstein breed, the descendants of different sire bulls.

MATERIALS AND METHODS

The material for research were first-calf cows of the local generation of the Holstein breed of the Joint-Stock Company "Aydyn", Komrat, Administrative and Territorial Unit Gagauzia, Republic of Moldova. First-calf cows are descendants of two breeding bulls: Form 999 (n = 14), Kiperush 79 (n = 11).

Exterior-constitutional features of first-calf cows were studied by taking measurements and calculating their constitution indices. The measurements of animals were carried out on 2-3 months after calving (Basovsky, 1983; Belozertsova, 2011). Body build indices were calculated according to the generally accepted method (Kostomakhin et al., 2007). The belonging of first-calf cows to different bulls was determined on the basis of analysis of the genealogical structure of the herd, using breeding certificates, breeding cards, artificial insemination logs and other documents of primary zootechnical registration. To study the productivity of cows, the data of primary zootechnical and breeding records were analyzed. Milk yield per lactation (305 days) was calculated on the basis of control milking.

The milk yield coefficient of cows was determined by the formula proposed by Startsev (1965): MC = MY/LW, where: MC - milk yield coefficient, kg; MY - milk yield for 305 days or shortened lactation, kg; LW - live weight, kg. The genetic potential of productivity of first-calf heifers was determined on the basis of the parental index of cows (PIC) according to the formula: PIC = (2M + MM + MF): 4, where: M - mother's productivity; MF is the productivity of the father's mother; MM is the mother's mother productivity. The realization of the genetic potential (RGP) was determined by the formula: RGP = actual productivity / expected productivity according to PIC x100%, where PIC - of the parental index of cows.

The relationship was determined between all indicators of the assessment of the conformation and milk production of first-calf cows by calculating the correlation coefficient (r).

Statical processing of research materials was carried out according to the methods of Plohinsky (1978), Merkuryev & Shangin-Berezovsky (1983). The data obtained in the course of the research were processed biometrically on a personal computer using Microsoft Excel programs; the reliability of the indicators was determined by Student.

RESULTS AND DISCUSSIONS

The exterior assessment of animals is an important component in a comprehensive breeding system. The results of studying the exterior of first-calf heifers of the local generation of the Holstein breed, the descendants of bulls Form 999, Kiperush 79, are presented in Table 1.

A comparative assessment of the exterior of first-calf heifers, descendants of bulls Form and Kiperush 79 showed that in some measurements the superiority between them was insignificant. On average for the sample, the height at the withers was 144.0 cm, the height at the croup was 148.5 cm, the oblique length of the body was 160.4 cm, the chest circumference was 197.5 cm, the width at the shoulder was 47.8 cm, pastern girth - 19.3 cm.

All evaluated first-calf heifers displayed a type characteristic for Holstein cattle, which is confirmed by the index assessment of their physique (Table 2, Figure 1).

It should be noted that the body in length was better developed at first-calf cows - the descendants of the bull Kiperush 79 - in terms of the index of elongation, they exceeded their peers by 1.0%.

Table 1. Exterior of first-calf cows of local generation of offspring of various bulls (X ± Sx)

Indices	Bull's daughters		Total, n = 41
	Form 999, n = 22	Kiperush 79, n = 15	
Height at withers	144.1 ± 0.9	143.6 ± 1.1	144.0 ± 0.6
Height at the croup	148.2 ± 0.9	149.0 ± 1.2	148.5 ± 0.7
Chest depth	74.7 ± 0.7	73.7 ± 0.9	74.3 ± 0.5
Chest width	42.4 ± 0.7	41.3 ± 0.9	41.9 ± 0.5
The width of the croup at the hips	48.0 ± 0.5	47.7 ± 0.74	47.8 ± 0.4
The width of the croup at the coxo-femoral joint	29.7 ± 0.4	29.4 ± 0.6	29.7 ± 0.3
Oblique length of the trunk	160.9 ± 1.5	160.7 ± 1.9	160.4 ± 1.1
Chest girth	198.7 ± 1.5	196.5 ± 1.7	197.5 ± 1.0
Pastern girth	19.3 ± 0.2	19.3 ± 0.2	19.3 ± 0.1

Table 2. Physique indices of daughters of bulls Form 999 and Kiperush 79

Indices	Form 999	Kiperush 79	Total	Dairy productivity direction (standard)
High-legged	48.2	48.7	48.4	46.5
Elongation	111.6	111.9	111.4	120.0
Pelvic breast	88.3	86.6	87.6	80.2
Breast	56.8	56.0	56.4	61.8
Consistency	123.5	122.3	123.1	118.0
Outgrown	102.8	103.7	103.1	107.0
Bone	13.4	13.4	13.4	14.6

Figure 1. First-calf heifers of the local generation of Holstein breed

In terms of pelvic and thoracic indices, the descendants of the Form 999 bull surpass their peers - the descendants of Kiperush 79, respectively, by 1.9 and 1.4%, the difference is not significant. The obtained index values were also compared with the milk-type standard. The value of the high-leg index for first-calf cows was on average 3.9% higher, and the breast index - 8.7% less than the standard for dairy cows. The obtained values of the overgrowth index indicate a flat topline at all analyzed animals. The bone index in comparison with the standard turned out to be on average 8.3% lower, which is due to the high height at the withers of Holstein cows.

It follows from the above data that first-calf cows, on average, have a relatively better development of the chest in depth, respectively, of the chest organs. Consequently, more developed chest organs provide a higher metabolism, which leads to higher milk production. This is confirmed by the analysis of the level of milk production for the completed first lactation (305 days) of first-calf heifers of the local generation - the descendants of bulls Form 999, Kiperush 79 (Table 3).

Table 3. Milk productivity of first-calf heifers of local generation - daughters of various bulls for the first lactation, (X ± Sx)

Daughters of a Bull	Number of cows, n	Milk yield		Fat		Live weight, kg	MC*, kg
		average per day, kg	for 305 days of lactation, кg	mass fraction, %	amount of milk, кg		
Kiperush 79	11	28 ± 0.4	8379 ± 118	3.87 ± 0.03	316 ± 7.5	595 ± 4.5	1409 ± 20.5
Form 999	14	28 ± 0.4	8534 ± 127	3.87 ± 0.03	330 ± 6.2	588 ± 3.4	1448 ± 21.9

MC* - of milk produced per 100 kg of live weight

As it can be seen from the materials in Table 3, the evaluated heifers exceeded the standard for milk yield and other indicators for animals of the Holstein breed. So, the milk yields of the daughters of the bull Kiperush 79 were 579 kg and of the daughters of the Form 999 bull - 734 kg of milk more than the breed standard. The fat mass fraction of the daughters of both bulls was by 0.27% higher than the breed standard. In terms of the amount of milk fat, the excess was 35 kg (daughter of Kiperush 79) and 49 kg (daughter of Form), the breed standard was 281 kg. A comparative analysis of the milk productivity of daughters of various breeding bulls showed that milk yield for 305 days of lactation at the daughters of the bull Form 999 is 155 kg more milk than at the daughters of the Kiperush 79 bull, the difference is not significant.

By live weight, first-calf heifers exceeded the breed standard (550 kg) by 45 and 38 kg, respectively, for daughters of Kiperush 79 and Form 999. The calculated milk production coefficient showed that for the daughters of both analyzed bulls, it exceeded the norm. Its value close to 1000 is considered normal. The daughters of the bull Form have the highest milk production coefficient - 1448 ± 20.5 kg of milk, slightly less - 1409 ± 21.9 kg - for the daughter of the bull Kiperush 79.

Analysis of selection and genetic parameters of economically useful traits of daughters from different bulls showed that the greatest coefficient of variability on average daily milk yield and milk yield per lactation was at daughters of Form 999, which is by 20.4 and 17.6 percent more, respectively, than at daughters of Kiperush 79 (Table 4).

Coefficient of variability on indices of content and amount of fat was higher at daughters of Kiperush 79 by 3.4 and 9.8 percent, respectively. Daughters of bull Form 999 had the greatest variability in average daily milk yield and milk yield per lactation, and daughters of bull Kiperush 79 had the greatest variability in content and amount of milk fat.

Table 4. Coefficient of variability of indicators of milk productivity (Cv, %)

Daughters	Average daily milk yield	Milk yield	Fat content	Fat amount
Kiperush 79	4.3	4.2	2.9	7.1
Form 999	5.4	5.1	2.8	6.4

Coefficients of milk yield variability are lower on average by 10-25%, fat content - 2.1-4.1%, milk fat - 11-25% than according to literature data. Thus, the descendants of bulls Form 999 and Kiperush 79, local generation cows, are more homogeneous with a reduced genetic diversity.

It was calculated the realization of the genetic potential in materials and methods of first-calf heifers of local breeding, whose mothers belonged to Dutch and German breeding (Table 5).

Table 5. Realization of the genetic potential of first-calf heifers of local breeding of various origins

Indicators		Dutch	German
Own productivity	milk yield, kg	8372±69,5	8479±98,8
	fat, %	3,82±0,02	3,84±0,03
Realization of genetic potential (RGP), %)	milk yield	91,4	92,1
	fat	94,1	97,9

Realization of the genetic potential of first-calf heifers of local breeding was at the level of 91.4-92.1% of the productivity of full-aged cows, with a content of fat of 3.82-3.89%, which is consistent with the results of data on Holstein cows of German breeding - milk yield of first-calf heifers was 90, 3% of the productivity of adult animals, with a content of fat of 3.95%, (GGI-Spermex. Uber Holstein. Population; Holstein Association USA, Inc)

For a more complete study of the nature of the relationship between milk productivity at cows of local breeding of the offspring of various bulls according to the 1st lactation, was done a study of the presence of a correlation between the milk yield, fat content in milk, live weight and measurements.

Table 6 shows the results of the correlation between the build of the exterior of cows for the first complete lactation with milk yield.

Table 6. Coefficients of interrelation of milk yield for 305 days of lactation - measurements of cows of local breeding, $r \pm m_r$

Correlated trait	Daughters	
	Kiperush 79	Form 999
Milk yield - height at withers	-0.143 ± 0.33	-0.217 ± 0.28
Milk yield - height at the sacrum	0.022 ± 0.33	-0.118 ± 0.27
Milk yield - chest depth	-0.318 ± 0.32	-0.501 ± 0.25
Milk yield - chest width behind shoulder blades	0.016 ± 0.33	-0.427 ± 0.26
Milk yield - width in hook bone	-0.145 ± 0.33	**0.462 ± 0.25**
Milk yield - width at ischial tubercles	-0.186 ± 0.33	0.032 ± 0.29
Milk yield - oblique body length	0.117 ± 0.33	-0.025 ± 0.29
Milk yield - chest girth behind shoulder blades	-0.176 ± 0.33	-0.207 ± 0.28
Milk yield - pastern girth	**0.324 ± 0.31**	-0.313 ± 0.27

It was found that the nature of the correlation of milk productivity with body measurements at daughters from different bulls has different meanings and the closeness of the relationship. Thus, the daughters of the bull Form 999 showed a moderate positive relationship between milk yield and width in hook bone (r = + 0.462). A weak negative relationship was found between milk yield and height measurements - height at the withers (r = - 0.217), height at the sacrum (r = - 0.118), as well as the girth of the chest behind the shoulder blades (r = - 0.207). A moderate negative relationship was established between the measurements of the depth of the chest and the width of the chest behind the shoulder blades - 0.501 and 0.427, respectively. A moderate positive relationship between milk yield and pastern girth (r = + 0.324) was found at the daughters of the bull Kiperush 79. However, according to the majority of measurements at the daughters of Kiperush 79, a weak negative correlation is observed: between milk yield and height at the withers (r = - 0.143), milk yield and width in hook bone (r = - 0.145), milk yield in the ischial tubercles (r = -0.186), chest girth behind shoulder blades (r = - 0.176).

The daughters of both analyzed bulls showed a positive moderate relationship between fat content and breast depth - +0.418 (Kiperush 79), - +0.451 (Form 999) (Table 7).

Table 7. Coefficients of the relationship between fat content and measurements of cows of local breeding, $r \pm m_r$

Correlated trait	Daughters	
	Kiperush 79	Form 999
fat content - height at withers	0.140 ± 0.33	0.051 ± 0.29
fat content - sacrum height	-0.117 ± 0.33	-0.031 ± 0.29
fat content - breast depth	**0.418 ± 0.30**	**0.451 ± 0.26**
fat content - width of the chest behind the shoulder blades	-0.088 ± 0.33	0.144 ± 0.28
fat content - width in hook bone	-0.251 ± 0.32	**0.360 ± 0.27**
fat content - width at tubercles	-0.429 ± 0.30	0.060 ± 0.29
fat content - oblique body length	**0.538 ± 0.28**	**0.170 ± 0.28**
fat content - chest girth behind the shoulder blades	0.159 ± 0.32	-0.049 ± 0.29
fat content - pastern girth	-0.435 ± 0.30	0.113 ± 0.28

Noteworthy is a noticeable positive relationship between the milk fat content and oblique body length in the daughters of the bull Kiperush 79 (r = + 0.538), as well as a moderate positive relationship between the fat content and the width in hook bone at the daughters of the Form 999 bull (r = + 0.360). For other measurements, there is a slight positive or weak negative relationship -0.031 (height at the sacrum) - for the daughter of the bull Form 999 to a moderate relationship -0.429 (width at the ischial tubercles) and -0.435 (pastern girth) - for the daughter of the bull Kiperush 79.

A weak negative correlation was revealed between live weight and height at the withers (r = -0.072), live weight - width in hook bone (r = -0.266) at the daughters of bull Form 999 (Table 8).

Table 8. Coefficients of the relationship between live weight and measurements of cows of local breeding, $r \pm m_r$

Correlated trait	Daughters	
	Kiperush 79	Form 999
Live weight - height at withers	0.369 ± 0.31	-0.072 ± 0.29
Live weight - - sacrum height	**0.533 ± 0.28**	0.071 ± 0.29
Live weight - chest depth	-0.017 ± 0.33	0.034 ± 0.29
Live weight - chest width behind shoulder blades	0.246 ± 0.32	0.360 ± 0.27
Live weight - width in hook bone	0.492 ± 0.29	-0.266 ± 0.28
Live weight - width at tubercles	**0.758** ± 0.22**	**-0.793** ± 0.17***
Live weight - oblique body length	-0.364 ± 0.31	0.307 ± 0.27
Live weight - chest girth behind shoulder blades	0.077 ± 0.33	0.244 ± 0.28
Live Weight - pastern girth	0.427 ± 0.30	0.174 ± 0.28

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

The value of the correlation between live weight and width at tubercles for the daughters of both analyzed bulls is high, however, the direction is different. So, for the daughters of the bull Kiperush 79, the relationship is positive - +0.758, for the daughters of the bull Form 999 - negative -0.793. The correlation coefficient "live weight - width at tubercles" is significant, at P<0.01 (daughter of Kiperush 79) and P<0.001 (daughter of Form).

Between the live weight and the height at the withers, width in hook bone and the girth of the pastern at the daughters of the bull Kiperush 79, the tightness of the connection is moderately positive and is +0.369, +0.492 and +0.427, respectively. At the daughters of the bull Form 999, the correlation between body weight and height at the withers, as well as the width in hook bone, is weak negative, respectively -0.072 - -0.266.

Thus, at daughters of Kiperush 79 and Form 999 have a moderate positive relationship between fat content and chest depth and oblique body length. It is noted a high multidirectional relationship between body weight and width in the ischial tubercles of daughters Kiperush 79 and Form 999.

It is known that the variability of the content and amount of fat in milk, as well as the live weight, depend on the variability of the milk yield of cows for lactation (Ivanova, 2018). The results of the study of the correlation between the performance indicators of daughters Kiperush 79 and Form 999 are shown in Table 9.

The relationship between milk yield and the percentage of fat in the milk of the daughters of the analyzed bulls was in a positive correlation from weak (+0.034, daughter of Kiperush 79) to moderate (+0.369, daughter of Form 999), which indicates the simultaneous selection for milk yield and fat content in milk.

Table 9. Correlation between indicators of milk productivity and live weight of cows of local breeding according to the 1st completed lactation, r ± m

Correlated trait	Daughters	
	Kiperush 79	Form 999
Milk yield - fat content	+0.034±0.33	+0.369±0.27
Milk yield - amount of fat	+0.373±0.31	**+0.935±0.10****
Milk - live weight	+0.188±0.33	-0.087±0.29

Note: *** P<0.001

It should be noted that there is a high correlation between the characteristics of milk yield - the amount of milk fat at the daughters of the bull Form 999 (+0.935) at (P<0.001), moderate - at the daughters of the bull Kiperush 79 (+0.373). A weak positive relationship was found between live weight and milk productivity at the daughters of the bull Kiperush 79 (+0.188), a weak negative (-0.087) - at the daughters of the bull Form 999. Low correlation coefficients between milk yield and live weight indicate a non-linear nature of the relationship between them, characterizes the homogeneity of the daughters of different bulls by live weight.

Thus, the correlation coefficients between milk yield and live weight (positive - daughters of bull Kiperush 79) and (negative - daughters of bull Form 999) indicate a non-linear nature of the relationships between them, and characterize the homogeneity of cows of the local generation of the herd of JSC "Aydyn" by live weight. Consequently, the revealed correlations between the studied traits make it possible to select cows according to their

exterior indicators, which contribute to an increase in milk productivity.

CONCLUSIONS

1. The body in length was better developed in first-calf cows - the descendants of the bull Kiperush 79 - in terms of the index of elongation, they exceeded their peers by 1.0%.
2. The obtained values of the overgrowth index indicate a flat topline in all the compared offspring of bulls of Forms 999 and Kiperush. 79. The bone index in comparison with the standard was on average by 8.3% lower, which is due to the high height at the withers of Holstein cows.
3. Daughters of bull Form 999 had the greatest variability in average daily milk yield and milk yield per lactation, and daughters of bull Kiperush 79 had the greatest variability in content and amount of milk fat.
4. Realization of the genetic potential of first-calf heifers of local selection was at the level of 91.4-92.1% of the productivity of full-aged cows.
5. The daughters of both analyzed bulls showed a positive moderate relationship between fat content and breast depth - +0.418 (Kiperush 79), - +0.451 (Form 999).
6. The correlation coefficients between milk yield and live weight - positive for daughters of bull Kiperush 79 and negative for daughters of bull Form 999, indicate a non-linear nature of the relationships between them, and characterize the homogeneity of cows of the local generation of the herd of JSC "Aydyn" by live weight.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

VARIABILITY OF THE RATIO BETWEEN A.M. AND P.M. MILK YIELD IN BULGARIAN MURRAH BUFFALOES UNDER TWO DIFFERENT FARMING SYSTEMS

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Abstract

The study assigned 65 buffaloes under intensive farming (FM-I) with 922 test day records, and 73 buffaloes in pasture system (FM-P) with 2505 records. The analyses of variance (LSMLMW and MIXMDL), including also year, season, parity and test day, showed significant effect of milking time on milk yield (P<0.001) and of test day and its co-effect with milker`s group on p.m./a.m. ratio in the herds from FM-P (P<0.001) and FM-I (P<0.05), respectively. The random factor individual was also highly significant on FM-P. On FM-I p.m. yield was averagely by 18.1% lower than a.m., the differences between a.m. and p.m. maintained practically constant throughout lactation. Atypical, contrary pattern was found on FM-P - p.m. by 22.3% higher than a.m. yield, the difference becoming smaller until seventh month. The effect of lactation persistency on FM-P (P<0.05) is expressed in inverse proportion to the p.m./a.m. ratio. The substantial variability of a.m.-p.m. productivity, in particular its peculiarities in separate herds of buffaloes, should be taken into consideration in the development of prediction models.

Key words*: buffaloes, test day, a.m., p.m. milk yield.*

INTRODUCTION

Farming practices have shown that the best milking frequency for dairy buffaloes is twice daily. As Thomas (2004) summarizes, buffalo udder has comparatively small cisternal area and fraction which affords three-time milking, so that the milk yield can possibly increase by 10%, as shown in the studies of Dash et al. (1976) and Ludri (1985). Nevertheless, this is not economical due to unjustified management and labor related costs, which in some regions forces farmer to milk their buffaloes even once daily (Borghese et al., 2007).

It was suggested that the pattern of morning-evening productivity in dairy animals is due to the diurnal circadian rhythm governed by the hypothalamus and the endocrine system, for which a specific day-and-night regulatory gene is responsible (Plaut & Casey, 2012). Like dairy cows, in the bubaline species this pattern of diurnal milk release is usually expressed in higher productivity from the morning milking and lower from the afternoon. On this basis, models for prediction of test-day and lactation milk yield have been developed for the purposes of selection (Khan & Akram, 1997; Peeva et al., 2009b).

The literature on the a.m.-p.m. rhythmicity in the buffalo productivity is scarce, including the few reports abroad of Akram & Khan (1996), Khan & Akram (1997), Gonzaga & Lorenzo (2007), Sahin et al. (2015) and that of Peeva et al. (2009b) from our previous research on the Bulgarian Murrah. Giving the sooner only indirect idea on the issue, these studies suggest great variation of the p.m./a.m. ratio among herds - from 0.5 to nearly 1.

The present aim was to study the variability of the ratio between a.m. and p.m. milk yield to characterize the pattern of diurnal productivity in buffaloes from two different farming systems.

MATERIALS AND METHODS

The study assigned milk yield test-day data about morning (a.m.) and afternoon (p.m.) milking obtained from the record books of two farms for the period from 2011 to 2018. As per Table 1, from farm 1 (FM-I) was used the information about 65 buffalo cows with

110 lactations, and from farm 2 (FM-P) - respectively 73 buffaloes with 280 lactations. On FM-I the buffaloes are bred intensively in a tie-stall barn with an exercise yard, and on FM-P they are also in a tie-stall barn in the night but on pasture (within a National Reserve) all through the day from April to October.

Table 1. Subsets of data per farm

Farm	Animals	Lactations	Test-day records	Records p.m.<a.m.
FM-I	65	110	922	799
FM-P	73	280	2505	691

The daily diet on FM-I from July to October involves 18 kg green foliage, 4 kg wheat straw, and 4 kg compound feed per capita, and from November the green roughage is replaced by 20 kg maize silage. On FM-P until October the buffaloes are fed 2 kg wheat straw, and 3.4 kg concentrate to supplement the pasture grazed, and from November - 3 kg alfalfa hay, 5 kg wheat straw, 4 kg compound feed, and 0.4 kg dried fodder beet chips.

The concentrate feed for both herds provides 1629 kcal energy and 96 g digestible protein and has the following composition: wheat - 15%, barley - 12%, corn - 56%, wheat bran - 10%, sunflower oilcake - 5%, dicalcium phosphate 0.6%, salt - 0.4%, and chalk - 1%.

On both farms the newborn calf is separated from the dam right after birth. The buffaloes are machine milked in cans twice daily - morning (a.m.) and evening (p.m.) - during which they are fed concentrate feed. In both herds the interval between milkings is 10 to 12 hours, depending on season. On FM-I milking is done in two groups, each of them served by a different milking operator, the two groups having been originally alloted on parity, productivity and temper basis. On FM-P the herd is served also by two operators milking the animals at random, not in personal groups.

The lactations that were used for data processing were chosen to have records at least to the seventh test day (only coupled a.m.-p.m. records) and for at least six test days. In this way, lactations with one missing test day record were also included, but in case of the sixth test day missing, the existence of seventh and eighth test day was required, and if the seventh was missing, the existence of eighth and ninth test day was necessary.

For the analysis of variance of milk yield per milking within each test day, datasets of 1844 records (double the test day records) for FM-I and 5010 for FM-P were processed. For that purpose, the software products LSMLMW and MIXMDL (Harvey, 1990) were used under the following overall model, herein referred to as MMY_{FM}:

$Y_{fq}= \mu+YR_g+SE_i+PA_j+TD_k+MT_q+e_{fq}$, where

- μ is the mean value of the trait;
- YR_g - the fixed effect of year of calving - in 2-year periods (g = 1…4);
- SE_i - the fixed effect of season of calving (i = 1…4);
- PA_j - the fixed effect of parity (j = 1…4);
- TD_k - the fixed effect of test day order (lactation month) (k = 1…6);
- MT_q - the time (a.m. or p.m.) of milking (q = 1…2); and
- e_{fq} - the residual effect.

For the analysis of variance of the ratio between milk yield from evening (p.m.) and morning (a.m.) milking for FM-I was used the following model, referred to as $AMPM_I$:

$Y_{fl}= \mu+YR_g+SE_i+PA_j+PER_l+PMY_m+ MG*TD_o+e_{fq}$,

where μ, YR_g, SE_i, PA_j and e_{fl} are as above;

- PER_l - the fixed effect of lactation persistency level (l = 1…4);
- PMY_m - the fixed effect of peak milk yield (m = 1…4);
- $MG*TD_o$ - the co-effect of milker's group and test day order (o = 1…12).

For that purpose, persistency index was calculated as the average of the ratios second/first to sixth/fifth test day.

The model for FM-P ($AMPM_P$) was like $AMPM_I$, but including the factor test day (TD_k) instead of the co-effect ($MG*TD_o$) and also using the random effect of individual, represented by the animal ear tag number (NO_f, f = 1...73).

The conventional statistical procedure (CSP) was also applied to a.m., p.m. yield and the ratio between them.

RESULTS AND DISCUSSIONS

The analyses of variance of milk yield per milking that resulted from model MMY_{FM}

within the separate herds are presented in Table 2. Normally, the environmental factors were found to affect milk yield - year and season of calving respectively at P<0.001 and P<0.01. The physiologically determined effects parity and lactation month (test day) are even better expressed (P<0.001), especially the latter.

Table 2. ANOVAs of milk yield per milking (a.m. and p.m. coupled), including F-test and P-value - model MMY_{FM}

Sources of variance	df	FM-I		FM-P	
		F	P	F	P
Year	3	50.91	0.0000	11.08	0.0000
Season	3	5.33	0.0013	3.93	0.0083
Parity	3	31.48	0.0000	17.53	0.0000
Test day	5	89.88	0.0000	679.16	0.0000
Milking time	1	311.14	0.0000	198.40	0.0000

Noteworthy is the significance of the factor milking time as a source of variation of the trait milk yield per milking on FM-P (F= 198.4, P<0.001) and especially on FM-I (F= 311.1, P<0.001), implying differences between a.m. and p.m. productivity.

Table 3 demonstrates the difference between the farms regarding the pattern of a.m.-p.m. milk yield. The buffaloes on FM-I manifest significantly higher yield from the morning milking and lower from the afternoon (P<0.001), while the situation on FM-P is opposite – a.m. yield lower than p.m. (P<0.001). On FM-I, evening milk yield constitutes 44.0% of the total test-day milk yield (a.m. + p.m.), while on FM-P the percentage is 53.4%.

Table 3. Conventional statistics of a.m. and p.m. milk yield and of the ratio between them

Trait	FM-I (n = 922)		FM-P (n = 2505)	
	$\bar{x} \pm S_{\bar{x}}$	CV	$\bar{x} \pm S_{\bar{x}}$	CV
a.m., kg	3.805 ± 0.047	37.8	2.858 ± 0.025	43.5
p.m., kg	2.996 ± 0.040	40.3	3.267 ± 0.029	43.7
p.m./a.m.	0.819 ± 0.011	40.5	1.223 ± 0.009	38.0

All between-farm differences and all within-farm a.m.-p.m. differences significant at P<0.001.

The values of the ratio between a.m. and p.m. milk represent this dependance, indicating that in the FM-I buffaloes p.m. milk is by 18.1% lower than a.m., and on FM-P evening milk yield is by 22.3% higher than morning.

In fact, as Table 3 also shows, on FM-I this proportion is on the basiis of significanly higher a.m. milk yield - by nearly 1 kg compared to FM-P. Respectively, the mean test-day milk yield (a.m. + p.m.) is also higher, and the difference between a.m. and p.m. productivity is two-fold, compared to FM-P. Relatively expressed, this difference constitutes 11.9% of the total milk yield per test day on FM-I, and 6.7% on FM-P.

It is also notworthy that both a.m. and p.m. milk yield and the ratio between them have very high variation on both farms - from 37.8 to 43.7%. This calls for analysis of the variance of the ratio - an issue that has not been treated to date.

Table 4 contains the weighed values (expressed as overall LSM means) from the analysis of variance of the p.m./a.m. ratio. They are little higher compared to those resulted from the conventional statistical procedure - 0.823 and 1.262 for FM-I and FM-P respectively - but still in keeping with the observed dramatic difference between the two farms.

Table 4. ANOVAs of p.m./a.m. ratio within the farms FM-I (model $AMPM_I$) and FM-P (model $AMPM_P$), including F-test and P-value

Sources of variance	df	FM-I (LSM = 0.823)		FM-P (LSM = 1.262)	
		F	P	F	P
Individual		-	-	1.85	0.0000
Year	3	4.50	0.0040	6.46	0.0003
Season	3	0.41	0.7474	0.67	0.5758
Parity	3	1.46	0.2218	0.14	0.9337
Test day (TD)	5	-	-	5.72	0.0000
Milker x TD	11	2.02	0.0236	-	-
Persistency	4	0.50	0.7342	2.60	0.0499
Peak yield	3	2.27	0.0780	2.47	0.0504

In the first place, the table presents the effects on the ratio within the farms. In the FM-P buffaloes the variation of the ratio is to a greater extent explained by the factors included in model $AMPM_P$, compared to FM-I. The factors ear tag number and test day order are highly significant on FM-P (P<0.001), implying substantial differences among the individual buffaloes and defining the lactation curve. Similarly, on FM-I test day has a

significant combined effect with milker's group ($P<0.05$).

In the buffaloes on pasture (FM-P), year of calving has pronounced effect, implying possible changes in management and nutritional conditions during the period of study, presumably variable condition of the available pasture. On FM-I, this effect is significant at $P<0.01$.

On the FM-I buffaloes the effects of persistency and peak yield are non-significant, while on FM-P they are marginal - respectively $P = 0.0499$ and $P = 0.0504$.

The results about the p.m./a.m. ratio in the intensively farmed buffaloes appear to be lower compared to the reported 93.4% by Peeva et al. (2009b) for the same herd, which is close the sooner to the finding for the Anatolian buffalo (Sahin et al., 2015). The ratio of FM-I is lower than the reported by Akram & Khan (1996) for the Nili-Ravi breed, and much higher than the value for Bulgarian Murrah buffaloes in the Philippines (Gonzaga & Lorenzo, 2007). More importantly, the buffaloes from FM-I are still in keeping with the commonly observed tendency for lower evening than morning productivity.

Nevertheless, the results about the farm on pasture are unprecedented - conrary to FM-I and to all other findings known to apply to the bubaline species. As Table 1 indicates, on FM-P the test-day records representing a typical pattern with p.m. milk yield lower than a.m. are only 27.6%, while on FM-I they are 86.7%. While the differences among the cited foreign studies might be due to different interval between morning and evening milking, this does not apply to the farms studied here.

The material of the study (Table 1) also shows that each FM-P buffalo participated with averagely 3.83 lactations. For comparison, on FM-I this number is 1.69 only, explaining to certain extent the relatively poorly fitted within-farm model. This is also implied in the higher total test-day milk yield of the intensively bred buffaloes, which is attributed to the problem with high incidence of short lactations on this farm and to the respective exclusions from that data subset.

On the basis of the significant effect of test day, Figures 1 and 2 represent the lactation curves from the a.m. and p.m. milking of the buffaloes from the two farms. In the first place, the figures demonstrate the difference between the farms, as per Table 3. The productivity of the FM-I buffaloes is presented by typical lactation curves (Figure 1), p.m. milk yield being lower than a.m. yield throughout lactation. The differences between morning and evening milk yield are practically uniform down to the seventh test day but, as related to the level of productivity, in fact the p.m./a.m. ratio declines.

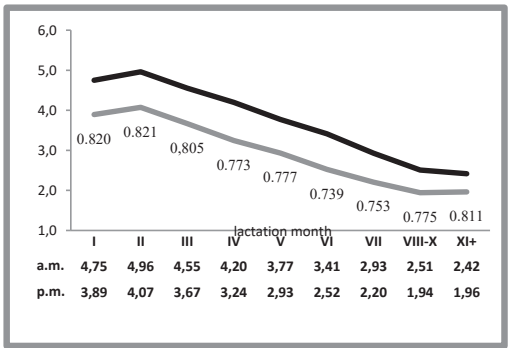

Figure 1. Lactation curves from a.m. (black) and p.m. (grey) milking on FM-I (by conventional statistical procedure), with the ratio bellow

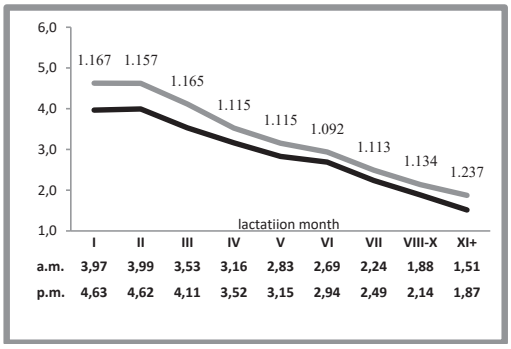

Figure 2. Lactation curves from a.m. (black) and p.m. (grey) milking on FM-P (by conventional statistics), with the ratio above

On FM-P the pattern is contrary – evening milk yield higher on all test days. The difference here, as Figure 2 graphically shows, is the faster decline in the p.m. compared to a.m. milk yield on this farm, rendering the difference between them diminishing to the seventh month. Nevertheless, despite this difference, there is also a decrease in the p.m./a.m. ratio, expressed mostly from fourth to sixth month. On both farms, in the last three months of the

normal (305 days) lactation, the ratio increases as compared to the previous months. It is even higher at the end of the long lactations (over 10 test days), especially on FM-P where the ratio is highest, mostly due to the low level of productivity at this stage.

Except on test-day milk yield (or a.m. yield in particular), lactation prediction models are developed on the basis of lactation curve, empirically expressed by persistency index (Khan et al., 2005; Peeva et al., 2009a).

Figure 3 represents the effect of level of persistency. On FM-I it is non-significant (P> 0.05) and expressed in inconsistent trend, the p.m./a.m. ratio being highest in the lactations with lowest persistency. In the buffaloes on pasture, the highest ratio belongs to the lactations with lowest persistency and, in general, the ratio decreases with the increase of persistency, i.e. the closer the range of daily milk yield throughout lactation, the closer the range between morning and evening productivity.

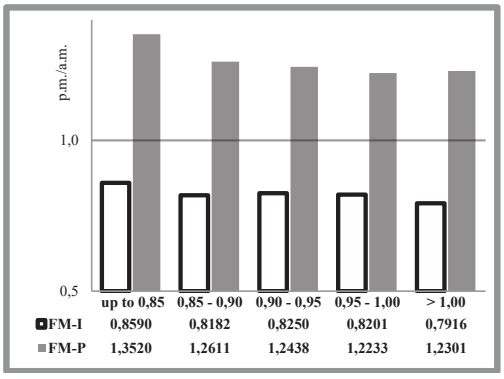

Figure 3. Effect of level of lactation persistency on FM-I (P>0.05, model $AMPM_I$) and FM-P (P<0.05, $AMPM_P$)

Presumably, since on FM-P the a.m. curve is more stable (persistent) than p.m., as seen in Figure 2, the persistency of total test-day milk yield is more dependent on the more unstable p.m. curve, i.e. the more persistent it is the smaller the difference with a.m. yield and the lower the p.m./a.m. ratio. In other words, the curve that is marked with lower productivity in this herd contributes more to the lactation persistency.

To summarize, the studied p.m./a.m. ratio showed to be highly variable within the studied farms, and to differ greatly among the previously established values in different herds worldwide (including FM-I and the cited sources), and especially between them and the atypical ratio in the FM-P buffaloes.

All this implies that the development of prediction test-day and lactation models in buffaloes should not blindly rely on partial records (usually a.m. milk yield) but take into consideration the possible peculiarities of the a.m.-p.m. ratio in separate herds.

CONCLUSIONS

The study established that in the FM-I buffaloes afternoon milk yield is averagely by 18.1% lower than morning, while on FM-P the pattern is atypical and contrary - p.m. by 22.3% higher than a.m. milk yield.

Test day is significant source of specific variance of the p.m/a.m. ratio in the FM-P buffaloes (P<0.001), and as a co-effect on FM-I (P<0.05). On FM-I the a.m.-p.m. difference stays practically constant throughout lactation, while on FM-P it becomes smaller down to seventh month, but in both cases the ratio declines.

The effect of persistency index on FM-P is significant (P<0.05) and expressed in highest p.m./a.m. ratio in the less persistent lactations, the ratio declining with the increase of the index. Year was found to be significant in both herds (P<0.01, P<0.001), while the other environmental factor season, peak yield and parity were not.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

CHARACTERISTICS OF THE KARAKACHAN SHEEP BREED REARED UNDER DIFFERENT CONDITIONS

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Abstract

Subject of the study were 682 sheep of the Karakachan breed, reared in three flocks in the region of the Middle and Western Rhodopes in 2018. The aim of the study was to create a phenotypic characteristic of the main selection traits of Karakachan sheep, under different breeding conditions in a mountainous area. The live weight and fertility traits were studied at 2.5, 3.5 and 4.5 years. Body measurements of sheep were taken at 2.5 years. The indexes for stretching, chest, massiveness and compactness were calculated. It was found that the Karakachan sheep breed meets the productivity and body measurements standards defined in the breeding program. The average live weight in the studied flocks varied from 36.620 kg to 39.410 kg under different conditions and altitude. The highest live weight was reached by the animals from the Agricultural and Stockbreeding Experimental Station (ASES) Smolyan - 40.53 kg at 4.5 years of age. The biological fertility for the studied period varied from 92.2% in ASES - Smolyan up to 103% in the village of Smilyan. Body measurements and body indexes establish the indicator dynamics, within the norm, under different breeding conditions and confirm the authenticity of the breed type in the Karakachan sheep breed.

Key words: *body indexes, body measurements, fertility, Karakachan sheep breed, live weight.*

INTRODUCTION

The economic conditions in the field of agriculture have imposed the tendency of breeding animals with lower requirements for breeding and feeding in areas with poor natural resources in recent years. This determines the increased interest in the local breeds of sheep, respectively in the Karakachan sheep breed. These animals are characterized by high vitality and adaptability, good disease resistance; lower but stable productivity and unpretentiousness to breeding conditions and feed resources. The local breeds are most suitable for the extensive conditions in the mountainous and semi-mountainous regions of the country. They play an important role in our agriculture, both economically and socially, and ecologically. This topic has been very relevant in connection with new trends in healthy nutrition and safety of animal products in recent years. These animals are reared in the absence of technological stress in a clean environment. The products obtained from them are of high quality, unique taste and indisputable benefits for consumers' health.

The Karakachan sheep breed is a typical local breed, which originates from the ancient breed Tsakel and its ancestor the European Mouflon. It is widespread on the Balkan Peninsula and in our country, mainly in the mountainous regions of the country. The peculiarities, the productive indicators and the condition of the Karakachan sheep population, reared in our country are the subject of studies made by Aleksieva (1979); Odzhakova (1994); Kafedjiev (1997; 1998); Odzhakova et al. (2002; 2020); Genkowski (2002); Panayotov et al. (2003); Nedelchev (2004); Vuchkov (2020).

Live weight is the main productive trait of sheep, an indicator of their development and physiological status. The possibilities for realization of a certain level of productivity depend on its dynamics. It is determined by a number of factors, including breed, sex, age, year of birth and production, type of birth, breeding technology and more (Aleksieva, 1978; 1987; Aleksieva et al., 1989; Vuchkov et al., 2008; Staikova et al., 2009). Fertility, along with live weight and growth intensity, are essential for the economic results of sheep breeding. Popova et al. (2007; 2015),

Odzhakova et al. (2010) found that from 40% to 80% of the revenues in sheep breeding in the various productive areas come from lamb sales. The main income is generated from the sale of lambs for meat in indigenous sheep breeding and sheep breeding for meat production. Staykova (2005) found that 69.34% of the relative share of income on a farm with Karakachan sheep is derived from meat income. Hinkovski et al. (1984) reported that in native sheep breeds the body measurements are one of the main selection traits, along with the type and condition of the animals. The body measurements are directly related to the level of productivity of the animals (Raichev et al., 1992). The analysis of the productivity of the animals during certain periods of time gives significant information about the correct direction of the selection process and the establishment of the factors that influence the full manifestation of the genetic potential. This motivates our research.

The aim of the study was to create a phenotypic characteristic of the main selection traits of the Karakachan sheep breed, under different breeding conditions in a mountainous area.

MATERIALS AND METHODS

Subject of the study were 682 sheep of the Karakachan breed, reared in three flocks in the region of the Middle and Western Rhodopes. Two of them were in the Municipality of Smolyan - the flock of the Agricultural and Stockbreeding Experimental Station (ASES) Smolyan - Group I, the village of Smilyan - Group II and one in the Municipality of Borino, the village of Borino - Group III. The survey was conducted in 2018. The data were obtained according to the standard methods and instructions provided in the Instruction for breeding and preservation of local (aboriginal) breeds in Bulgaria (2003). The live weight and fertility traits were studied at 2.5, 3.5 and 4.5 years. Body measurements of ewes at 2.5 years were made. Wither height, Diagonal length of the body, depth and chest width were measured with a Lidtin rod. Chest girth, whistle scope and tail length were measured with tape. The indexes for stretching, chest, massiveness and compactness were calculated. Statistical data processing was performed using software version 20 of the SPSS program.

The farm of Agricultural and Stockbreeding Experimental Station-Smolyan is located at 1170 m above sea level. Pastures at an altitude of 1000-1200 m were used to feed the animals. The soil was brown forest type, highly rocky and had a clayey-sandy composition in the conditions of mountain-forest climate. The supply of humus was low. The reaction of the soil was acidic pH = 4. The grass composition of natural grasses consisted of 70% cereal grasses, about 9% legumes and 21% other grasses. The farm was well provided with concentrated feed for winter feeding.

The farm in the village of Smilyan used pastures from 950-1100 m above sea level, with predominantly brown forest type soils. In the composition of the grassland the highest was the percentage of cereal grasses - about 36%, followed by legumes - 35% and other grasses - 29%. The favourable ratio between the three grass classes was a prerequisite for good protein, energy and mineral supply of the nutritional needs of sheep.

The farm in the village of Borino is located at 1100m above sea level, used mountainous pastures located on brown forest and cinnamon forest type soils. The content of cereal grasses was about 64%, followed by legumes - 14% and other grasses - 22%.

RESULTS AND DISCUSSIONS

Data on live weight at different ages during the study are shown in Table 1. The highest live weight was represented by the sheep from the flock of ASES - Smolyan (Group I), which increased with age and reached a maximum of 40,530 kg at 4.5 years ($P<0.001$). At 2.5 years of age we had a statistically significant difference of 1.58 kg between groups I and II and 1.39 kg between groups I and III. At 3.5 years the difference was 1.74 kg, between groups I and II and 3.07 between groups I and III. At 4.5 years of age, the same differences increased to 4.96 kg, and 4.29 kg ($P<0.001$). The variation level within group I was low, 6.8% for live weight at 2.5 years to 9.2% at 3.5 years. Animals from the other two groups showed lower and close in values average live weight by age. The total average weight by flocks for the studied period was the highest in ASES -Smolyan - 39.410 kg ($P<0.001$), which

was explained by the good conditions of breeding and feeding, ensuring the realization of the genetic potential of the breed on this trait. The results obtained by Kafedzhiev (1997) for live weight at 2.5 years (38.630 kg) for the same flock of the Karakachan breed were close to the values obtained by us. The established live weights at different ages in group II did not follow a certain trend. The variation of the trait was more significant at the end of the age period, which was probably due to environmental factors. The values of the trait in group III did not indicate significant differences in the weight of the different age groups. The low variability degree for groups I and III (less than 10%) was due to the long-term selection in terms of own productivity. The data indicated good equalisation for the studied trait in the flocks of this breed. The obtained results for average live weight of the Karakachan breed from groups II and III were close to those reported by Hinkovski et al. (1984), Kafedzhiev et al. (1992), Genkovski (2002) and for the flock of group I the results were analogous to the study of Panayotov et al. (2003). The analysis of the data on live weight by ages in the three flocks of the Karakachan breed gave information about the condition of the breed and the phenotypic manifestation of the genetic potential of the animals reared under different conditions and altitude.

Table 1. Live weight of sheep from three flocks (kg)

Karakachan sheep breed	Age															
	Live weight at 2.5 years				Live weight at 3.5 years				Live weight at 4.5 years				Total average			
	n	**x̄**	**± Sx**	**C%**	**n**	**x̄**	**± Sx**	**C%**	**n**	**x̄**	**± Sx**	**C%**	**n**	**x̄**	**± Sx**	**C%**
ASES-Smolyan I group	103	38.45**a**	± 0.229	6.8	103	39.23**a**	± 0.327	9.2	33	40.53**a**	± 0.251	7.8	239	39.41**a**	± 0.201	8.8
Smilyan village II group	73	36.87**b**	± 0.264	8.7	73	37.49**b**	± 0.231	10.7	37	35.97**b**	± 0.414	17.5	183	36.78**b**	± 0.367	9.6
Borino village III group	144	37.06**c**	± 0.339	7.7	83	36.16**c**	± 0.356	8.9	33	36.64**c**	± 0.471	7.78	260	36.62**c**	± 0.219	6.8
** Significance p<0.01 *** Significance p<0.001		a: b*** a: c***				a: b*** a: c*** b: c **				a: b*** a: c***				a: b*** a: c***		

Another important selection trait is fertility, which largely determines the economic effect of animal husbandry. Table 2 shows the biological fertility of sheep from the three age groups - at 2.5, 3.5 and 4.5 years. The results from group I for the obtained average number of lambs were close, respectively 0.922, 0.950 and 0.963 number of lambs per ewe. During the second lambing period, the mothers from group II gave 106% fertility compared to 2.5 years old and 101% compared to 4.5 years old. The values of the trait in group III followed the tendency to increase with age. The differences of 5% between 2.5 and 3.5 years and 7.3% between 2.5 and 4.5 years had no statistical significance. In the analysis of the data for the biological fertility of the three groups of the Karakachan sheep breed, it was established that during the study period the animals from the village of Smilyan (group II) had the highest fertility. The variation coefficients ranged from 32 to 45%. Similar results were obtained by Staykova et al. (2015) for the Karakachan sheep breed, where the variation coefficients ranged from 26.36% to 39.61%.

Table 2. Fertility of sheep from three flocks

Karakachan sheep breed	Age											
	Biological fertility Number of lambs/sheep at 2.5 years				Biological fertility Number of lambs/sheep at 3.5 years				Biological fertility Number of lambs/sheep at 4.5 years			
	n	**x̄**	**± Sx**	**C%**	**n**	**x̄**	**± Sx**	**C%**	**n**	**x̄**	**± Sx**	**C%**
ASES - Smolyan I group	103	0.922	± 0.036	38	103	0.950	± 0.031	32	33	0.963	± 0.107	27
Smilyan village II group	73	0.976	± 0.044	35	37	1.030	± 0.079	41	37	1.022	± 0.116	39
Borino village III group	144	0.931	± 0.112	41	83	0.980	± 0.105	45	33	1.004	± 0.121	34

In the case of aboriginal sheep breeds with local significance, the body measurements are an important indicator, embedded in our and foreign programs for conservation of genetic resources (Nedelchev et al., 2014). They largely characterize the bodily characteristics of animals, the dynamics of change in individual parts of the body, as well as their abilities in terms of productivity. The body measurements are one of the main controlled traits, alongside the type and condition of the animals. The results of the three farms are shown in Table 3.

Table 3. Body measurements in a Karakachan sheep of different farms

Body measurements	Smolyan - I n - 103		Smilyan village - II n - 73		Borino village - III n - 144		Significance
	$\bar{x}$	± S $\bar{x}$	$\bar{x}$	± S $\bar{x}$	$\bar{x}$	± S $\bar{x}$	
Wither height, cm	55.24 a1	± 0.655	52.95 b1	± 0.444	54.69 c1	± 0.359	a1: b1 p<0.01 c1:b1 p<0.01
Diagonal length of the body	57.97 a2	± 0.663	56.14 b2	± 0.306	56.75 c2	± 0.689	a2: b2 p<0.01
Depth of the chest, cm	25.25 a3	± 0.163	24.43 b3	± 0.159	24.93 c3	± 0.167	a3: b3 p<0.001
Width of the chest, cm	17.60 a4	± 0.173	16.52 b4	± 0.195	16.38 c4	± 0.234	a4: b4 p<0.001 a4: c4p<0.001
Girth of the chest, cm	80.22 a5	± 0.825	69.76 b5	± 0.681	69.48 c5	± 0.306	a5: b5 p<0.001 a5: c5 p<0.001
Scope of the whistle, cm	8.29 a6	± 0.106	7.55 b6	± 0.207	7.84 c6	± 0.157	a6: b6 p<0.5
Tail length, cm	26.1 a7	± 0.347	24.59 b7	± 0.405	24.92 c7	± 0.303	a7: b7 p<0.001 a7: c7 p<0.001

The sheep from ASES - Smolyan were characterized by higher values for wither height, diagonal length of the body, depth and width of the chest. The wither height of the Karakachan sheep in groups I and III were close in value, respectively 55.24 and 54.69 cm, it was lower in group II - 52.95 cm (P<0.01). For the second indicator - diagonal body length, the sheep from group I were 1.83 cm longer than those from the village of Smilyan - group II (P<0.01) and 1.22 cm superior to the animals in the village of Borino - Group III. The same trend was maintained in the indicator for chest depth (P<0.001). In terms of chest girth, the highest average value was shown by the animals measured in ASES - Smolyan - I group (80.22 cm). This indicator was 14.1% higher than group II and 15.4% higher than group III (P<0.001). In terms of the whistle range, the difference between groups I and II was 0.74 cm in favour of the first (P<0.001). The average length of the tail in sheep in ASES - Smolyan (Group I) was 1.51 cm larger than Group II and 1.18 cm larger than Group III (P<0.001). The results obtained in our study for body measurements were close to the results obtained by Sedefchev et al. (2011) and indicate that the Karakachan sheep breed in the studied flocks is characterized by a well-preserved authentic breed type.

Body indexes reflect the relationship between two or more body measurements related anatomically or functionally and expressed in %. They characterize the proportions of the body and change during growth and development of animals. Through them the growth is controlled and the deviations from the norm are established. The body stretching index expresses the ratio between the length and height of the body. This index varies slightly with age. The results (Figure 1) show that most stretched were the animals from group II - 106%. The values are close in group I - 105% and group III - 103%.

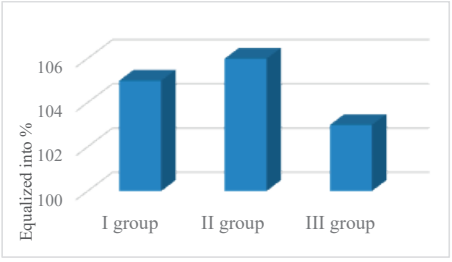

Figure 1. Stretching index

The chest index (Figure 2) shows the relative development of the chest, its width and shape. The results obtained for groups I, II and III are close, respectively 69%, 67% and 67%.

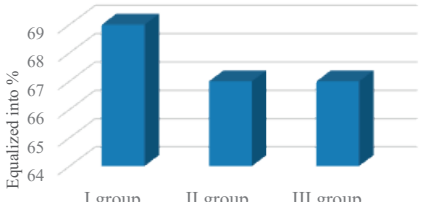

Figure 2. Chest index

The massiveness index (Figure 3) shows the relative development of the carcass. The Karakachan sheep from group I surpass by 9.8% those from group II and by 14.2% those from group III.

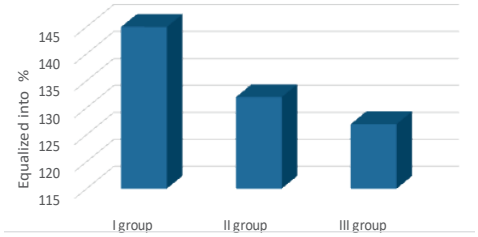

Figure 3. Index of massiveness

The compactness index (Figure 4) expresses the ratio between chest girth and body length. This index is related to body weight and characterizes the compactness of the body by supplementing the chest index. The lowest values are in group III 122%.

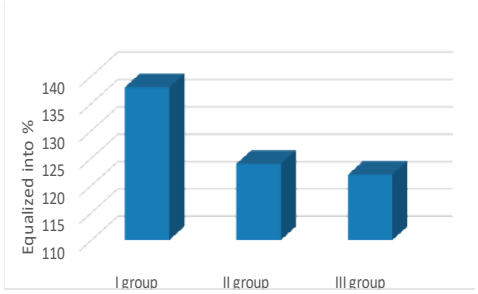

Figure 4. Index for compactness

The Karakachan sheep from group I are superior by 11.2% to the sheep from group II and by 13.1% to the sheep from group III according to this index. The results for the body indexes reflect the differences in growth and development of body proportions due to the different environmental factors in the three farms. The calculated values of the indexes are within the normal range for the authentic type of Karakachan sheep breed, reported by Odzhakova et al. (2020).

CONCLUSIONS

The Karakachan sheep breed, reared under different conditions in a mountainous area, meets the productivity and body measurements standards defined in the breeding program.

The average live weight in the studied flocks varied from 36,620 kg to 39,410 kg under different conditions and altitude. The highest live weight is reached by the animals from the flock of ASES Smolyan - 40.53 kg at 4.5 years.

The biological fertility for the studied period varied from 92.2% in the flock of ASES-Smolyan to 103% in the village of Smilyan.

Body measurements and body indexes showed the dynamics of the indicators, within the norm, under different conditions of breeding and confirm the authenticity of the breed type in the Karakachan sheep breed.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

RESEARCH REGARDING THE EVALUATION OF THE CURRENT STATUS OF A NEW SHEEP POPULATION CREATED IN ROMANIA

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Abstract

The research aimed to make a more complex assessment of the current breed status of a new sheep population with very good milk production skills. The population concerned represented the result of research activity to form a new breed with superior skills in milk production. The biological material was represented by a significant number of 1200 females and 58 breeding rams. This herd is part of the living stock and is registered in the Genealogical Register. The data processing was done by methods accepted by the experimental technique. From the data processing, it results that the actual size of the new type population is placed at the level that allows the application of a sustained selection program. For this indicator, the average values show that the new population is evolving very well in other areas, where the pedoclimatic conditions are slightly different from those in the training area. The average value of the actual size varies depending on the holding and the area, from the minimum of 105.88 for the populations outside the training area to 115.16 for the analyzed herd in the training area.

Key words*: Awassi sheep, breeding isolation, crossbreeding, improvement, Rovasi, Ţigaie.*

INTRODUCTION

In Romania, Awassi sheep were imported especially for making crossbreeds with local breeds. The first import of Awassi parents was made from Israel in May 1973. The imported flock was brought to the Research and Development Institute for Sheep and Goat Breeding Palas-Constanța and was represented by 10 rams and 70 females, aged for 7 months. Subsequently, this herd was transferred to the Rușețu Sheep and Goat Breeding Research and Development Station - where it was kept purebred for several generations.

Simultaneous with the herd imported from Israel, it was used at various crosses with local Romanian breeds, a process that ended with obtaining new breeds of sheep. Thus, at the research-development unit from Palas-Constanța, by crossing with Merinos de Palas and Friza, the Palas Milk Breed was formed, which was approved in 2010, and at the Research and Development Station for Raising Szekler Sheep and Goats - Bacău by crossing with the rusty variety of Țigaia breed, a new type of sheep was obtained, called Rovasi, which is in the process of homologation.

Rovasi is a population of a new type of sheep that provide large milk production, being selected and raised in purebred for several generations. The new type of sheep was formed, evolved, and developed in the northeastern part of Romania, in an area populated with traditional Romanian sheep breeds, represented especially by Țurcană and Țigaie, with all varieties of color, but also by their crossbreeds.

The new genotype created is well adapted to the harsh environmental conditions in the breeding area and provides superior milk production, located very close to the level reached by specialized breeds. It also shows a well-established organic resistance and is suitable for growth based on the application of different technologies.

This population has different characteristics from the old and traditional breeds in the formation area (Ţigaie and Ţurcană) but is similar to most breeds in which Macedonian Awassi, Egyptian Baladi, Ausi, Nuami,

Gezirieh, and others (Aziz et al., 1988; Dzabirski et al, 2016; Fareed et al., 1981; Enas El-Hady, 2020; Goot, 1986; Gürsoy et al., 1993; Galala et al., 2008; Hamdon, 1980; Juma et al., 2006; Kassem et al., 1980).

MATERIALS AND METHODS

The biological material was represented by females and males in the living stock of the new sheep population existing in different breeding ponds. The entire research staff is registered in the Genealogical Register.

The actual size of the new breed was analyzed on a numerically significant population, consisting of 1200 females (adult and young breeding sheep) and 58 breeding rams, using the calculation below:

$$Ne = \frac{4NmxNf}{Nm + Nf},$$

where:

Nm = number of males,

Nf = number of females.

Based on the value determined for the actual size of the population, it was possible to highlight the homozygous growth rate for each new generation of animals (ΔF) and the genetic effect due to inbreeding, knowing that ΔF = 1/2 Ne.

To determine the current breeding isolation status of the herd belonging to the new population, data from the genealogical register were used.

Based on them, the value of the reproductive isolation coefficient was calculated, and the formula used was the one described by Wright, 1921, quoted by Drăgănescu, 1972; Drăgănescu, 1979:

$$CIR = \frac{AA - (AI + II)}{AA + AI + II},$$

where:

CIR = reproductive isolation coefficient;

AA = the number of individuals admitted to the range, studied from the core nucleus and having both native parents;

AI = the number of individuals admitted to the range, studied from the core nucleus and having one native parent and another immigrant;

II = the number of individuals admitted to rang, studied from the core nucleus and having both immigrant parents.

To estimate the genetic distance between the individuals of the new sheep population, the standard method described by Nei (1972) was based on the construction of dendrograms containing data organized in subcategories until the desired level of detail was reached. Dendrogram was constructed using the neighbor-joining (NJ) method (Saitou and Nei 1987). Nei's standard distances (Ds; Nei, 1972), observed heterozygosity (Ho) and expected heterozygosity (He), neighbor-joining trees, and bootstrap values were computed using the DISPAN computer package (Ota, 1993).

To evaluate the existing inbreeding degree, the parent-descendant chain was analyzed because the degree of kinship is given by the position in the pedigree of the common ancestor of two or more individuals.

RESULTS AND DISCUSSIONS

In full agreement with the main objective, the methodology used in the formation of new breeds was based on the application of a systematic cross-breeding program which aimed at summing on a new type genotype the gene pool responsible for milk production capacity (from Awassi) and keeping the breed local genes responsible for organic resistance, adaptation to different technological conditions, resistance to climatic factors and different pathogens etc.

To fix the production characters, but also to increase the degree of genetic similarity, the half breeds obtained in the R_2 generation were subjected to a sustained selection process, being retained only individuals who had a correct external appearance and a body conformation that include the basic requirements for the type of sheep with high milk production.

According to the work scheme used to create the new population (Rovasi), an absorption cross was constantly applied until the R_2 generation was obtained, when the gene pool in the new half breed was 78.70% owned by the Awassi breed and only 12.30% of the Ţigaie breed.

From that moment on, the R_2 and R_3 individuals were reproduced for 4 generations (back cross), applying a rigorous selection, and a controlled reproduction where, the goal was represented by increasing the degree of phenotypic similarity, fixing, and consolidating the specific characters of the new type of sheep. Currently, the nucleus is closed from a reproductive point of view for over seven generations, and on this background, the degree of similarity between them has increased; also, the characteristics of production, reproduction, and those that give a high resistance to pedo-climatic factors specific to the training area have been consolidated.

The actual size was determined to objectively highlight the current status of the new population, knowing that the strength of the genetic drift effect is governed by the size of the actual population.

When the actual population size is small, the genetic drift will be stronger.

Performing mathematical calculations allows highlighting the fact that the values of the actual size of the population vary depending on the farm and area, from a minimum of 105.88 to populations outside the training area to 115.16 to the number analyzed in the training area.

As these differences are very small, it can be stated that there is a good extension of the new type of sheep outside the training area.

Based on the value determined for the effective size (Ne) of the population, it was possible to highlight the growth rate of homozygosity on each new generation, applying in the calculation of the inbreeding coefficient the mathematical calculation principles presented by Lush (quoted Vintilă, 1988).

Determining the inbreeding coefficient is very important in the analysis because it helps to identify the status of a population, highlighting the proportion of decreasing homozygous locus compared to the base population, due to the use of related breeding.

Determining the degree of inbreeding involves the use of computational procedures that measure the proportion of homozygous genes of an individual from related parents. The calculation procedure starts from the premise that the degree of kinship between two individuals is given by the pedigree position of the common ancestor or ancestors.

Thus, for the calculation of the degree of inbreeding, it is necessary to know the number of generations that link the parents of the analyzed individual to the common ancestor. There are several methods for determining the degree of inbreeding: the free generation method, the removal method, the inbreeding coefficient, the Hardiman method, and the matrix method of table evaluation (Lush, 1967; Sas et al., 2004).

Applying the mathematical calculation relations to determine the inbreeding coefficient on the analyzed population, it results that regarding the Rovasi breed, on the entire analyzed herd, a value of 6.38 was obtained, tensing in the moderate type of inbreeding.

The practical importance of determining the inbreeding coefficient is because the fact that it serves to estimate the degree of kinship that can also be estimated on an analysis based on pedigree.

Table 1. Actual size, sex ratio, and inbreeding rate

Specification	Males (Nm)	Females (Nf)	Sex ratio Nm/Nf	Actual size (Ne)	Inbreeding coefficient (ΔF)	Breeding isolation index
Inside the creation area	30	715	23.83	115.16	5.31	0.77
Outside the creation area	28	485	17.32	105.88	6.48	0.81
Total population	58	1200	16.21	221.30	6.38	0.79

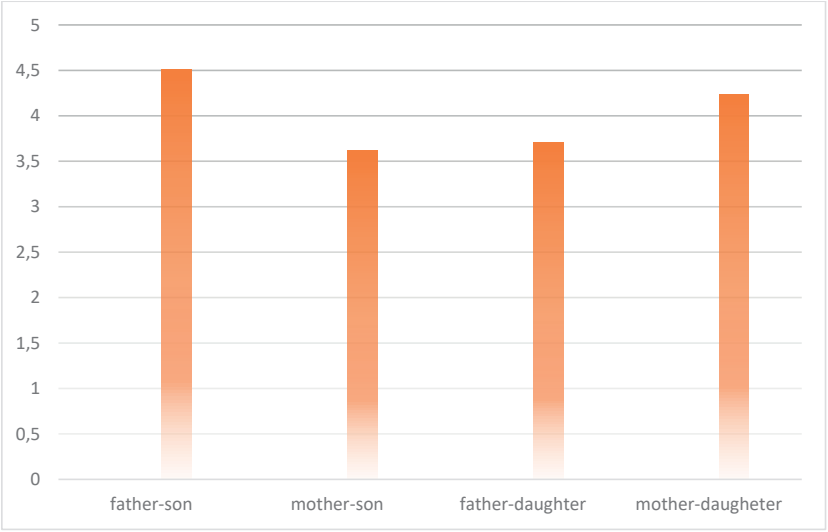

Figure 1. The interval between generations at Rovasi breed

This genetic indicator is extremely important because the calculation of the proportion of homozygous genes of an individual from genetically similar (related) parents can be done with the help of the inbreeding coefficient (Fx) made by Wright. The inbreeding coefficient measures the increase in the proportion of homozygous loci in inbred individuals compared to the base population.

Determining the degree of reproductive isolation is a basic objective of research because the mechanisms of reproductive isolation are dependent on evolutionary processes, behavioral processes, and many physiological processes critical to species.

Reproductive isolation prevents members of different species from producing offspring by crossing them or ensures that the resulting offspring are viable and sterile (Beker, 1959; Barton et al., 1986; Strickberger, 1978; Futuyma, 1998).

At the species level, through the mechanisms through which it acts, reproductive isolation creates certain barriers that maintain the integrity of the fundamental characters that define a given population by reducing the flow of genes from other related populations.

From the determination of this indicator a slight difference of the average values from the two growth basins can be observed. The fact that in the training area the reproductive isolation index is 0.77 shows that the claims and restrictions regarding the penetration inside the population of some breeders of wide breeds are stricter.

The analysis of the intergenerational interval is an extremely important indicator of selection, representing an essential factor of improvement, directly influencing the effect of selection on each new generation.

This character is important in the improvement work because it conditions the speed or the rhythm of obtaining the effect due to the selection. (Pipernea, 1979; Popa, 2006).

Based on the analyzed data, it is found that for the newly created breed the interval between generations has a variable average duration, being 4.2 on the mother-daughter chain and decreases to 3.6 on the mother-son chain.

In the case of the analysis performed on the father's chain, a contradictory evolution is registered because the interval between generations is longer, respectively 4.51 for the father-son relationship and is reduced to 3.7 for the father-daughter relationship.

Comparing these values with those determined by other authors it can be seen that the interval between generations is located at the same coordinates with the mention that the one for father and son and father and daughter has lower average values because it is found that farmers show a desire to introduce more early sheep in the reproductive and productive circuit.

Comparing these values with those determined by other authors it can be seen that the interval between generations is located at the same coordinates with the mention that the one for father and son and father and daughter has lower average values because it is found that farmers show a desire to introduce more early sheep in the reproductive and productive circuit.

CONCLUSIONS

The research aimed at analyzing the current status of a sheep population in the final phase of approval as a new breed of sheep.

The analyzed population was formed in the pedo-climatic conditions specific to the northeastern area of Romania through systematic crosses of the Awassi breed and local Țigaie sheep from the rust variety.

The determination of the effective size of the population has an average value of 221.30, a level that supports the application of an efficiency improvement program based on rigorous selection activities to improve performance and performance.

The determination of the inbreeding coefficient on the analyzed population indicates that the average value of 6.38 allows the inclusion in the moderate type of inbreeding.

The value of the reproductive isolation index registers close values, being 0.77 in the training area and 0.81 outside this area, which indicates some difference between these areas in terms of the requirement for the penetration of some breeders from the population of late rase.

The interval between generations has a variable average duration, being 4.2 on the mother-daughter chain and decreases to 3.6 on the mother-son chain; in the case of the analysis performed on the father's chain, a contradictory evolution is registered because the interval between generations is longer, respectively 4.51 for the father-son relationship and is reduced to 3.7.

ACKNOWLEDGEMENTS

The research carried out was funded by the Ministry of Agriculture and Rural Development, through the ADER 2020 Sectoral Plan, cod project 8.1.7.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

STUDY REGARDING THE FACTORS INFLUENCING THE PRICE AT PSI ANNUAL AUCTION

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Abstract

At PSI Auction, the annual performance sales that take place is presented to the international clients an exclusive number of young horses for training or jumping over obstacles, destined for the biggest international equestrian competitions. This paper aimed to examine all the horses participating in the auctions in 2019 and 2020, on Eurodressage, taking into account the economic aspect of the 3 factors: horse's sex and year of birth, and the equestrian discipline. The alpha value was set at 0.05 for all statistics. Following the analysis of the results, regarding the year of birth (all participants in this study being young horses), the sex of the horses, and also the equestrian discipline there were no significant differences. In conclusion, the auctioned horses, whether they are mares, stallions, or gelding, whether the year of birth or breed, after being selected for participation in the auction, horses are sold at very high prices and will be capitalized according to the buyer's interest in the reproductive direction, as well as for sports, being trained for the biggest competitions in the world.

Key words: *auction, equestrian, horses, price.*

INTRODUCTION

Performance Sales International, the world-renowned auction, has been held annually for more than 40 years in early December (Ashenfelter & Genesove, 1992; Seow et al., 2005). The Kasselmann family has been associated with the land they live on since the 12th century. The property was first a farm and was originally owned by the Count of Tecklenburg in the 16th and 17th centuries. In 1765 Frederick the Great allowed farmers to buy land from the authorities. The Kasselmann family took advantage of this opportunity, and the family members became free farmers from that point on.

The farm was not run only as a direct farm: hospitality has always been a key component of operations, and during the summer holidays the inhabitants of the city went to visit the farm, the concept is now widespread and known as "farm holidays" (Eklof & Lunander, 2003).

During the twentieth century, changes in agriculture also shifted Hof Kasselmann's goal to the only current focus: the sport of training.

Performance Sales International was founded in 1980 when equestrian legends George Morris, Franck Chapot, and Paul Schockemöhle came together during the Baltimore World Cup final and conceived the idea of an innovative horse trading company. Businessmen have arrived in the United States to sell horses from the largest breeding areas in Europe (Reed, 2008).

They chose young, promising, well-trained horses that could easily fit the American training and competition circuit (Dubois & Ricard, 2007; Dulugeac, 2005; Lungulescu & Tăblie, 1999).

The international clients, currently not only from the USA but from all over the world, are following with interest the annual PSI auction, meeting in Ankum, Germany (Ashenfelter, O. and Genesove, D.).

The horses for the upcoming PSI auction are identified and carefully selected months in advance in the stables with experienced staff in Kasselmann and Schockemöhle, the presentations of these horses being eagerly awaited by potential buyers (Bennet, 1986; Bowling et al., 2000; Dulugeac, 2005)

The annual international performance sales within PSI Auction take place in December, the international clientele being presented with an exclusive selection of young horses for training

or jumping over obstacles, destined for the biggest international sports competitions (eurodressage.com).
The annual international performance sales within PSI Auction take place in December, the international clients being presented with an exclusive selection of young horses for training or jumping over obstacles, destined for the biggest international sports competitions.
Newport of Rhode Island was the winner of the first edition of the PSI Auction, which has now reached its 42nd edition (psi-auktion.de)
When the demand for this auction concept appeared in Europe, after only 2 years, Performance Sales International settled in Germany, finding that this country is perfect. Thus, the auction center in Ankum is a location preserved for this day.

MATERIALS AND METHODS

1. Analyzed horses - for this research, there were 70 sport horses examined, sold in the annual PSI auction for 2019 and 2020 (History - P.S.I. Auction). The distribution of horses for the three variables considered (sex, year, and discipline) is presented in Table 1.

Table 1. The distribution of horses regarding the sex, year, and discipline

Discipline	Sex			Year	
	stallion	mare	gelding	2019	2020
Dresage	15	16	11	21	21
Jumping	11	7	10	7	21

2. Statistical analysis - the data were statistically processed using SPSS Version 21 for Windows (IBM, USA), the following analyzes being performed:
- Independent T-Test;-Descriptive statistics regarding the distribution of variables, average, median, graphs, etc.
Data were interpreted using Excel 2007 (Microsoft) and analyzed using SPSS Version 21 for Windows (IBM, USA).
The purpose of the statistical analysis was to explore any differences in the price obtained at the PSI auction for sport horses differentiated according to year, sex, and discipline. The Alpha value was set at 0.05 for all statistical tests.

RESULTS AND DISCUSSIONS

The selling price obtained in the PSI auction, for the 70 horses studied ranged between 30000 and 1300000 euro, with an average of 339821.43 euro in 2019 and between 72000 and 1600000 euro, with an average of 346857.14 euro in 2020 and between 30000 and 1300000 € (Figure 1).

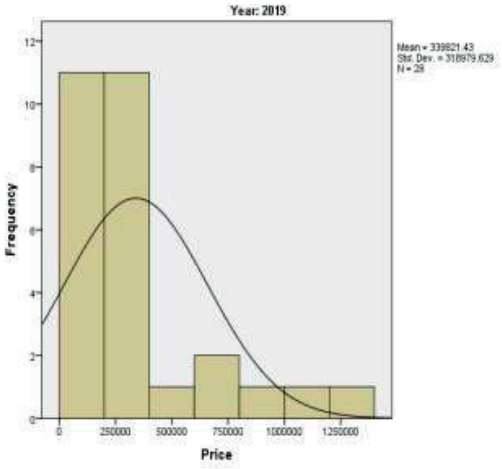

Figure. 1. The usual distribution of price for the studied population in PSI auction (2019)

The results show that the selling price obtained in the PSI auction, for the 42 horses studied varies between 72000 and 1600000 euro, with an average of 346857.14 € in 2020 (Figure 2).

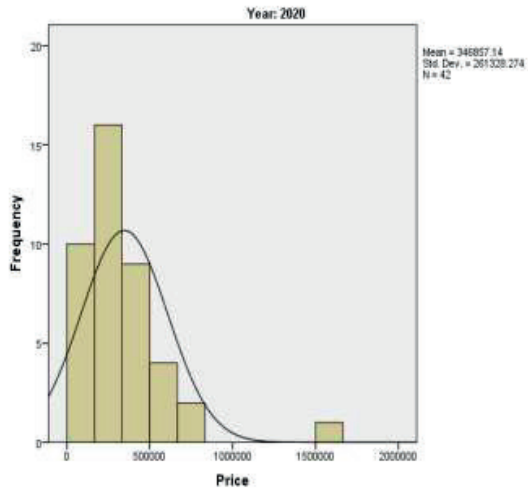

Figure 2. The usual distribution of price for the studied population in PSI auction (2020)

The results show that for:
• Gender variable: - 32.9% of horses are mares, 37.1% are stallions and 30% are geldings
• Variable year: - 40% for 2019 and 60% for 2020.
• Discipline variable: - 60% of horses compete in training and 40% compete in show jumping.

1. Factor sex
For the sex variable, the results of the statistical analysis show that the selling price obtained in the PSI auction for the 70 horses studied does not show a statistically significant difference for the level of significance 0.05 (F=0.204, p = 0.816).
Thus, in the descriptive statistics for mean, the values are close: for mares = 369739.13 euro, for stallions = 345192.31 euro, for geldings = 314476.19 euro (Table 2).
We can say that there are no statistically significant differences between mares, stallions and geldings.
Given the rigorous selection procedure and the criteria that future PSI participants must meet, there are no statistically significant differences in terms of sex.

Table 2. Statistic data of variable sex of horses (euro)

Sex of the studied horse	N	$\bar{x}$	s	$\pm s_{\bar{x}}$	Min.	Max.
Mare	23	369739.13	350223.723	73026.694	72000	1600000
Stallion	26	345192.31	260178.250	51025.153	86000	1300000
Gelding	21	314476.19	237031.141	51724.436	30000	955000
TOTAL	**70**	**344042.86**	**283559.642**	**33891.860**	**30000**	**1600000**

The Figure 3 indicates the results regarding the price obtained at PSI auction for the 70 studied horses after the sex criteria. It can be seen that the price drops from 369739.13 euro (mares) to 314476.19 euro (geldings), the stallions being in between (314476.19 euro).

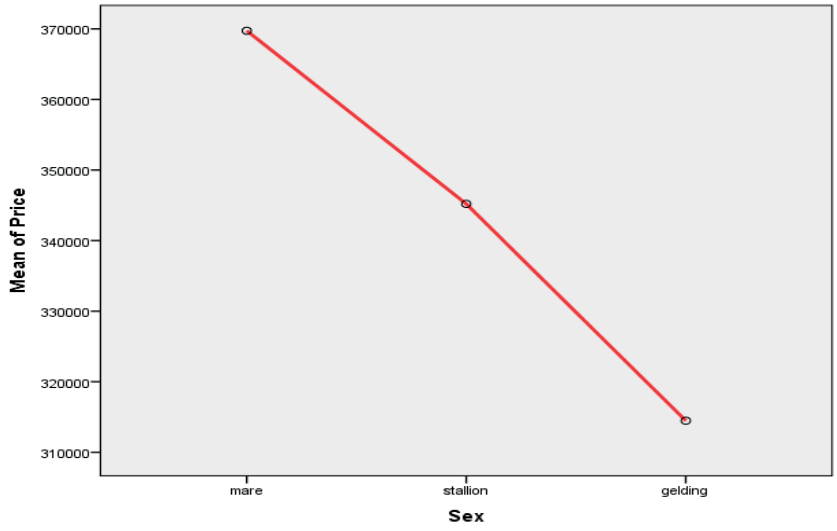

Figure 3. Mean of price of variable sex (euro)

2. Year factor
For the year variable, the results of the statistical analysis show that regarding the sale price obtained in the PSI auction for the 70 horses studied, there are no statistically significant differences for the significance level of 0.05 (t = -0.101, p = 0.920). We can say that no there are statistically significant differences between the two years studied. Also the identified mean was 339821.43 euro for 2019 year and 346858.14 euro for 2020 year (Table 3).

Table 3. Statistical data for the year factor

Sex of the studied horse	N	$\bar{x}$	s	$\pm S_{\bar{x}}$
2019	28	339821.43	318979.629	60281.484
2020	42	346857.14	261328.274	40323.828

The distribution of percent for variable year, is presented in Table 4 where 32.9% of the total was represented by mares, 37.1% by stallions and 30% by geldings; it was observed that the lower values of bootstrap for percent were: 21.4% for mares, 25.7% for stallions and 18.6% for geldings. On the other hand, the upper values were: 44.6% for mares, 48.6% for stallions, and 40% for geldings. This means that for mares it can be stated with confidence of 95% that variation ranged between 21.4-44.3 for mares, 25.7-48.6 for stallions and 18.6-40 for geldings (Table 4).

Table 4. The percent of sex for variable year

Sex of the studied horse	N	Percent	Bias	Std. Error	Bootstrap for percent*	
					Lower	Upper
Mare	23	32.9	-.2	5.5	21.4	44.3
Stallion	26	37.1	.2	5.6	25.7	48.6
Gelding	21	30	-.1	5.4	18.6	40.0
TOTAL	**70**	**100**	**0**	**0**	**100.0**	**100.0**

*Unless otherwise noted, bootstrap results are based on 1000 bootstrap samples.

In Table 5 it is represented the distribution of percent for 2019 and 2020, where the first year had a frequency of 28% and the second one a frequency of 42%, indicating lower values of 28.6% for 2019 and 48.6% for 2020, and upper values of 51.4% for 2019 and 71.4% for 2020. It can be stated with confidence of 95% that variation ranged between 28.6-51.4 for 2019 year and 48.6-71.4 for 2020 year.

Table 5. The percent for 2019 and 2020

Year	N	Bias	Std. Error	Bootstrap for percent*	
				Lower	Upper
2019	28	-2	5.8	28.6	51.4
2020	42	-2	5.8	48.6	71.4
TOTAL	**70**	**0**	**0**	**100.0**	**100.0**

*Unless otherwise noted, bootstrap results are based on 1000 bootstrap samples.

The distribution of equestrian sports like dressage and jumping are presented in Table 6 where the lower values of bootstrap are 48.6% in dressage, and 30% in jumping; the upper values were 70% for the first discipline, and 51.4% for the second one. So it can be stated with confidence of 95% that variation ranged between from 48.6-70 for dressage discipline and from 30-51.4 for jumping (Table 6).

Table 6. The percent for dressage and jumping

Year	N	Bias	Std. Error	Bootstrap for percent*	
				Min.	Max.
Dressage	42	-.3	5.6	48.6	70.0
Jumping	28	.3	5.6	30.0	51.4
TOTAL	**70**	**0**	**0**	**100.0**	**100.0**

*Unless otherwise noted, bootstrap results are based on 1000 bootstrap samples.

3. Equestrian discipline factor

For this factor, the results of the statistical analysis show that the results regarding the sale price obtained in the PSI auction for the 70 horses do not show statistically significant differences for the significance level of 0.05 (t = -0.077, p = 0.939). We can say that there are no statistically significant differences between the two disciplines. Also the mean for dressage was 341904.76 euro and 347250.00 euro for jumping (Table 7).

Table 7. Statistical data for the equestrian discipline factor

Sex of the studied horse	N	$\bar{x}$	s	$\pm S_{\bar{x}}$
Dressage	42	341904.76	277627.388	42838.836
Jumping	28	347250.00	297357.700	56195.323

CONCLUSIONS

In conclusion, we can say that the annual PSI auctions have carried out over time, in the 42 editions, very rigorous selections of the participating horses.

Thus, the top specimens, which promise the best sports performances, are carefully selected in the selection centers, in Kasselmann and Schockemöhle, months before the auction.

Following the analysis of the results of the horses in the PSI auctions in 2019 and 2020, there were no significant differences between the years, all participants in this study being young horses.

The results obtained from the statistical analysis show that the selling price obtained in the PSI auction for the horses studied does not show statistically significant differences for the level of significance 0.05 (t = -0.101, p = 0.920). We can say that there are no statistically significant differences between the two years studied, 2019 and 2020.

Following the analysis of the results of the horses from the PSI auctions in 2019 and 2020, there were no significant differences for the sex of the participating horses that would influence their sale price in the auction. Thus, for the sex variable, the results of the analysis vary between 72000 euro and 1600000 euro, with an average of 346857.14 euro for 2020. The results of data processing show that the selling price obtained in the PSI auction for the 70 horses studied does not present a statistically significant difference for significance level 0.05 (F = 0.204, p = 0.816).

Following the analysis of the results of the horses participating in the PSI auctions in 2019 and 2020, there were no significant differences in their discipline, training, or jumping that would influence the sale price in the auction. The results of the statistical analysis show that the selling price obtained in the PSI auction for the subjects studied does not show statistically significant differences for the level of significance 0.05 (t = -0.077, p = 0.939).

Thus, in the descriptive statistics for the mean for factor sex, the values are close: for mares = 369739.13 euro, for stallions = 345192.31 euro, for geldings = 314476.19 euro.

Also we can state that there are no statistically significant differences between the two years studied regarding the factor year. Also the identified mean was 339821.43 euro for 2019y and 346858.14 euro for 2020y. This means that for mares it can be stated with confidence of 95% that variation ranged between 21.4-44.3 for mares, 25.7-48.6 for stallions and 18.6-40 for geldings.

These horses, whether they are mares, stallions, or geldings, regardless of the year, or breed, are sold at very high prices and will be used according to the interest of the buyer both on the reproductive line, those active in terms of

reproduction, as well as on the sports line, being trained for the biggest competitions in the world.

We can say that there are no statistically significant differences between the two disciplines. Also the mean for dressage was 341904.76 euro and 347250.00 euro for jumping.

During the auction, horses with high-performance genealogical bloodlines are presented for sale, which have been very carefully selected in advance by the sales team.

We can conclude that in the PSI auctions the most promising horses are chosen and carefully selected both for the eventuality of reproduction and for sports competitions, and precisely this analysis and rigorous selection can be the reasons why there are no significant differences in their selling prices.

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History - P.S.I. Auction (psi-auktion.de)

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P.S.I. Auktion (https://psi-auktion.de/)

HOF KASESELMANN - From agricultural holding to equestrian hub! (https://hof-kasselmann.de/en/about-us/history/)

HOF KASESELMANN - Dresage horses a variety oh different auctions (https://hof-kasselmann.de/en/auctions/)/

Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

GENETIC DETERMINISM FOR THE MASTITIS RESISTANCE IN ROMANIAN PINZGAU

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Abstract

The Pinzgau breed in Romania (known as Pinzgau from Transylvania) is a very valuable genetic resource, and can be found mainly in the NW of the Carpathian Mountains, the Dornelor basin with the Bucovina High Hills (Obcinele Bucovinene), SW of Transylvania. In the absence of a coherent active conservation program, this resource may disappear at any time. In our opinion, the major vulnerability of the Pinzgau breed is the indiscriminate infusions with Red Holstein or Simmental, as a consequence of the lack of firm actions to preserve the genetic heritage. The objective of this study was to estimate genetic parameters for milk somatic cells count, character that significantly affect the health of animals and milk constituents that gives its quality. Also, this character could allow a selection of individuals in the direction of resistance to mastitis. A total 252 milk yield and associated characters records, belonging to 63 females from Suceava County, which coming from 8 sire families, for 4 lactations were analyzed. In the absence of consistent data, the paternal origin of females was established after ear tags. The method used for genetic parameters estimates was REML. Study has revealed the existence of a poor genetic determinism for somatic cell count and a high variability of character analyzed according to number of lactation. The results indicate that environmental factors have a greater contribution to the phenotypic manifestation of character. So, the number of somatic cells must be exclusively an indicator of milk hygiene and not included in selection for mastitis resistance of Romanian Pinzgau within active conservation program.

Key words*: genetic parameters, Romanian Pinzgau, somatic cells count.*

INTRODUCTION

The Pinzgau breed is also called Pinzgauer after its region of origin (Pinzgau, near Salzburg, Austria) and is an alpine breed. The breed emerged in the 19th century from the local alpine breeds and was developed in three directions: traction, milk and meat. Its use as traction animals has played an important role in the history of the breed, contributing to the increase in size, the development of muscles and exceptional walking skills. Pinzgau traction oxen were famous in the lowlands of Austria, Bavaria, and neighboring countries.

Around 1820, Pinzgau specimens were exported to countries such as Romania, Yugoslavia, the Czech Republic and Slovakia, and later to over 25 countries around the world.

In last decades, even in the birthplace, the number of specimens of the breed declined drastically due to changes in "fashion" and intensive agriculture, which caused the race to be in danger. Pinzgau, only Austrian indigenous breed, worldwide famous, should receive special attention through the establishment of national park and through the use of race to achieve its organic productions.

In Romania, the Pinzgau breed is treated as an independent breed. In Romania there are flocks that are made up of the descendants of Pinzgau cattle brought on the territory of the country during the Habsburg Empire.

The breeding area of the Pinzgau cattle breed in Romania is quite large, covering a part of the north west of the Carpathian chain, the Dornelor basin with the Bucovina High Hills (Obcinele Bucovinene), the south west of Transylvania and not only.

There were about 35000 Pinzgau cattle at the national level.

In Romania, Red Pinzgau breed formed after absorption crosses made between local breeds of cattle (Grey Steppe and Mocăniţa) and Pizgau of Austria, since the second half of the nineteenth century, and black Pinzgau named "Cow of Dorna" by crossing local cattle with various mountain improved breeds (Pinzgauer, Mölltal, Zillertal, Dux-Zillertal, Dutch, Brown, etc.).

The Pinzgau breed is exploited today in two directions: for milk production (quantitatively lower than the Austrian and German Simmental, but close in terms of quality) and for the special quality of the meat. It is very well adapted to severe environmental conditions, suitable for extensive exploitation. These are the main reasons why the breed should be kept in a form of active conservation.

The Transylvanian (Romanian) Pinzgau breed from is in real danger of being absorbed by the Simmental super-breed, of which Bălţata Românească (Romanian Spotted) is also a part.

Kladlecik et al. (2004) state that the Pinzgau breed has reached an endangered status, a consequence of the sharp decrease in numbers over time. According to Draganescu (2003), Transylvanian Pinzgau breed from seems to be only vulnerable. As long as there is a very low chance that the situation will change in the growing areas, the breed has all the chances to be preserved in the next 15-20 years. However, the author states that during all this period it is mandatory to build an efficient conservation program.

As is known, one of the major steps in the design of the breeding or active conservation program is to determine with maximum accuracy the population genetic structure. Many of the decisions to be taken in animal breeding, in relation to the choice of breeding system and selection methods, depend on the values of genetic parameters. The accuracy of the genetic parameter estimation depends on the amount and quality of the primary data and the statistical model selection (Grosu, 2005; Popa, 2009).

It is known that the quality of milk can be affected by the number of somatic cells. These can provoke some change in the milk constituents. Also, the somatic cells count is a tool for assessment milk health by diagnosing of sub-clinic mastitis and can be a trait that allows the selection of individuals for genetic resistance to this disease.

Mastitis is an inflammation of the mammary gland, being a pathology that relatively frequently affects dairy cows, causing, in addition to issues related to the ethics of cow exploitation, numerous and important economic losses.

From this point of view, the selection of dairy cows for mastitis resistance should be included in the breeding program, but the difficulty of this action lies in the weak genetic determinism of this trait (Urioste et al., 2010; Koeck et al., 2012).

Over time, selection for mastitis resistance has been used both directly and indirectly (de Haas et al., 2002; Odegard et al., 2002).

In general, resistance to disease has a weak genetic determinism, being strongly influenced by environmental factors. As a result, the characters on the viability and resistance to disease are difficult to improved, giving preference to environmental conditions improving (Popa, 2009) i.e. milking hygiene. For clarification, studies on the genetic structure of the population associated with this character, become binding. In this regard, research has shown that direct selection for mastitis resistance is totally inefficient, a consequence of the very weak genetic determinism of this pathology (heritability between 0.02 and 0.05) and the difficulty in measuring character (Mrode & Swanson, 1996; Rupp & Boichard, 1999). On the other hand, the inclusion of udder health in the selection criteria of dairy cows becomes mandatory to compensate for the negative effect of selection for milk quantity on health, longevity and reproduction (Oltenacu & Broom, 2010).

A number of studies have shown that the inclusion of somatic cell counts in dairy cows breeding programs for indirect selection for mastitis resistance was superior compared to those that included only the milk yield, a superiority quantified by their overall economic value (Rogers, 1993; Colleau & Bihan-Duval, 1995). The issue is supported by genetic correlations between somatic cell number and milk quantity, ranging from 0.13 to 0.22 (Rupp & Boichard, 1999; Carlen et al., 2004).

Indirect selection for mastitis resistance uses as a selection criterion a character that has a

higher genetic determinism than the manifestation of the disease itself, namely the number of somatic cells (Rupp & Boichard, 1999).
In the case of indirect selection, there must be a close genetic correlation between the primary and the secondary character in order for it to have the expected effect. Research has shown a genetic correlation between 0.7 and 0.8 between the number of somatic cells and the appearance of mastitis in its clinical form, and selection to decrease the number of somatic cells should increase resistance to mastitis (Shook & Schutz, 1994; Rupp & Boichard, 1999; Kadarmideen & Pryce, 2001).
In general, research on the genetic determinism of somatic cell numbers has shown a low heritability, with heritability values of the average number of somatic cells per lactation ranging from 0.05 to 0.17 (Odegard et al., 2002; Carlen et al., 2004).
We note that in many countries, the selection for udder health is made not only on the number of somatic cells, but also on a variant of this character obtained by logarithmic (logarithmic transformation) in order to normalize the distribution, the new feature called somatic cells score (Guzzo et al., 2018).
In this context, the objective of this study was to estimate genetic determinism for Transylvanian (Romanian) Pinzgau milk somatic cells count, using a methodology that gives the maximum accuracy in conditions of the existence an inconsistent data.

MATERIALS AND METHODS

In order to estimate genetic parameter values, data collected by authors from cows from individual households, with known and identifiable origin, were used. To analyze parameters in dynamic were included in the analysis only females presenting records to an equal number of lactations.
A total 252 milk yield and associated characters records, belonging to 63 females from Suceava County, which coming from 8 sire families, for 4 lactations were analyzed. In the absence of consistent data, the paternal origin of females was established after eartags.
The method used for genetic parameters estimates was REML developed by Sir Ronald Fisher (1925) and perfected by Patterson and Thompson in 1971.

RESULTS AND DISCUSSIONS

The results on the average performance of milk somatic cells count are presented in Table 1.

Table 1. Descriptive statistics for somatic cells count

Spec.	UM	n	$\overline{X} \pm s_{\overline{X}}$	s	v%
Lactation 1	no/ml	63	389606 ± 19279.1180	153076.1974	39.29
Lactation 2	no/ml	63	309416 ± 13062.4991	103716.2432	33.52
Lactation 3	no/ml	63	286753 ± 15291.0857	121411.2202	42.34
Lactation 4	no/ml	63	375242 ± 19348.1202	153624.0748	40.94

The data presented in Tables 1 shows that the average values of milk somatic cells count are characteristic of a population with a large variability, within standards for cattle milk quality, and that trait can have a good response to selection. The values of the descriptive statistics indicate the existence of a population that can constitute object of a breeding or active conservation program, with a sufficiently large field for action of artificial selection.
However, although the values obtained for the number of somatic cells are in the standard for cow's milk, they suggest the existence of conditions for the maintenance of cows that would need to be improved.
Being a local population of cows, from individual households, so small farms, the housing of cows is debatable in terms of meeting the welfare conditions. Especially on rainy days, most likely, the mud is taken to the stable, and the resting area of the animals becomes unsuitable. These conditions certainly affect the health status of cows. Improper housing conditions will increase the chance of developing diseases in the mammary gland, creating the opportunity for the development of infections. To all this is added a poor milking hygiene.
As a result, it becomes imperative to improve farm management, in order to maintain well-being (clean, dry stables with a comfortable rest area, with the possibility of easy removal of manure) and ensure impeccable milking hygiene. Improving environmental conditions, in addition to animal welfare issues, will result

in a reduction in its variation and, as a result, the share of additive variance in the total phenotypic variance will increase and heritability will be higher, ensuring the premises of efficient selection.

Heritability is defined as the rate of additive genetic variance in the phenotypic variance. Estimates of heritability coefficients are considered very important as indicators of a breeding program effectiveness. Heritability coefficient values for the somatic cells count, for 4 lactations, are presented in Table 2.

Table 2. Heritability values for somatic cells count

Spec.	Lactation 1	Lactation 2	Lactation 3	Lactation 4
	$h^2 \pm S_{h^2}$	$h^2 \pm S_{h^2}$	$h^2 \pm S_{h^2}$	$h^2 \pm S_{h^2}$
Somatic cells count	0.065 ± 0.205	0.084 ± 0.250	0.172 ± 0.184	0.106 ± 0.290

The analysis of data presented in Table 2 shows that the milk somatic cells count is a character that has a low genetic determinism, along the 4 lactations analyzed. The variation of heritability coefficient from one age to another can be explained by the existence of different polygenic complex that is involved in genetic determinism of somatic cells count, environmental conditions influence, errors due to sample size, or errors on production recording.

Heritability coefficient values for somatic cells count found in present paper are similar to those reported by other authors. Heritability for somatic cells count varied between 0,05 and 0,29 (Coffey et al., 1985; Kennedy et al., 1982; Monardes et al., 1985; Monardes et al., 1985; Mrode & Swanson, 1996; Mrode et al., 1998). Jattawa et al. (2012) found a heritability value for somatic cells count of 0.12 and although the heritability was low, authors suggest that the trait could be improved by selection, but in conjunction with improvements in farm management.

The heritability values for somatic cells count found in present paper showed that exist some other factors (specified above) which were more important than additive factors. So, at first side, it appears advisable that the female selection for somatic cells count in order to improve mastitis resistance should not be done.

Even if the value of the heritability of the somatic cell number is small, still the economic importance of this character, seen through the prism of the economic losses generated by mastitis, claims the need to improve it through selection. Certainly, we expect the response of the population to the selection to be small.

As a result of this state of affairs, two solutions can be discussed: on the one hand, the selection of females to focus on other traits associated with milk production, and for the resistance to mastitis, respectively for the decrease of the number of somatic cells, to improve the hygiene conditions in the farms, and on the other hand the selection for the number of somatic cells to be included in the breeding program, but along with improvements in farm management, respectively in hygiene and maintenance conditions. Opting for the second variant, taking into account the genetic determinism of the character, there is the chance to reduce the environmental variation, increase the value of heritability and thus make the selection more efficient. However, the issue is difficult to implement in the case of a local population, respectively at the level of small breeders. Improvements are also needed in performance control to obtain consistent data.

Genetic correlation between characters is another important aspect of establishing the selection objective within breeding or active conservation program. The genetic correlation values for the somatic cells count with other milk production traits, for 4 lactations, are presented in Table 3.

Table 3. Genotypical correlation estimates between somatic cells count and other traits

Specification	Lactation 1	Lactation 2	Lactation 3	Lactation 4
Somatic cells count x				
-Milk yield	0.088	-0.122	0.068	0.060
-Fat yield	0.276	-0.181	-0.148	-0.220
-Protein yield	0.188	-0.200	0.229	0.198

The results presented in Table 3 show that the somatic cells count is weakly and negatively correlated with milk yield in the second lactation and very weakly and positively in the other analysed lactations.

The values of genetic correlations differ from those reported by other authors (Rupp & Boichard, 1999; Carlen et al., 2004) who communicate values of this parameter between 0.15 and 0.22. The small values of genetic

correlations found in this paper are determined by the genetic structure of the population, but can also be attributed to a sample error caused by the small data set.

The negative genetic correlation recorded in the second lactation between the number of somatic cells and the milk yield seems to be convenient for the selection of females. Thus, this negative value indicates that cows with high production would tend to have a low number of somatic cells, so with a low chance of mastitis, which is beneficial if maintained regardless of the degree of lactation, which does not happen.

Negative genetic correlations between somatic cells counts and fat yield suggest that selection for the latter should lead to fewer somatic cells.

Thus, with the exception of the first lactation, a simultaneous selection made in the logic of increasing the amount of fat and decreasing the number of somatic cells could have a favorable effect on the overall economic efficiency of the breeding program, which would translate into an increase in farmers' incomes, provided that the results are confirmed on large, consistent data sets.

Also, a smaller amount of protein could lead to a smaller number of somatic cells. Similar results were reported by Schutz et al. (1990). On the other hand, according to market demands, selection for a larger amount of protein would lead to increase the number of somatic cells and, very likely, the incidence of mastitis. This situation requires improvement of the environment conditions, especially hygiene of milking, because the amount of protein in milk is an important character which directly affects the quantity and quality of cheese.

CONCLUSIONS

The somatic cells count, as a component of cattle milk quality, at Transylvanian (Romanian) Pinzgau analysed females, has enough genetic variation for selection, but the low genetic determinism suggest that environmental factors have a greater contribution to the phenotypic manifestation of the trait.

As a result, resistance to mastitis, quantified by the number of somatic cells, could be included in the breeding program, but without being mandatory, at least in a first phase. Ensuring proper maintenance conditions, even imposing a minimum standard of well-being in individual households, would have the effect of reducing environmental variation and increasing the value of heritability, which would streamline selection for udder health.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

PRESERVATION OF RAM SEMEN BY REFRIGERATION

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Abstract

The research was performed on ram semen. The biological material was the Moldovan Karakul rams. Ejaculates with a mobility of over 70% and a sperm concentration of over 2 billion / ml were allowed for processing. The basic medium used was SȚJ (sucrose, sodium citrate, egg yolk). An additional component introduced in the basic environment was experienced the biologically active preparation LB / MP obtained from yeast from the brewing by the Institute of Microbiology and Biotechnology of the Academy of Sciences of the Republic of Moldova. After diluting and microscopically testing the sperm using the CEROS computer system, the experimental batches were introduced into the cold room at + 2-4 degrees. Mobility assessment was performed at 24 hours intervals. Sperm mobility after 120 hours of storage by refrigeration was maintained at 68.0 ± 5.8 % in the experimental group where the concentration of the preparation in the base medium was 0.8%, mobility allowed for inoculation. Based on the experimental results obtained, it was proposed to dilute and preserve ram semen by refrigerating the SȚJ medium supplemented with 0.8% biologically active preparation LB/MP.

Key words*: ejaculate, mobility, ram, refrigeration, sperm.*

INTRODUCTION

The sensitivity of the sperm cells leads to the obligation that in all the time spent outside the natural conditions, ie between the time of harvesting and sowing, all measures must be taken to protect the sperm from harmful agents on its viability (Ivanova et al., 1999; Tardif et al., 1997).

If the temperature decreases suddenly, the thermal play is installed, which results in an increase in the permeability of the cell membrane and the consequent loss of the proteins of potassium lipids and lipid phosphorus (Ladha et al., 1999).

Thermal shock can be felt even by diluted semen, although diluents provide considerable protection against sudden drops in temperature (Mircu, 2001; Perez-Pe et al., 2010; Tulcan et al., 2004).

In the process of diluting the sperm with dilution media and preserving by refrigeration there are structural changes of the sperm that lead to the deregulation of the transmembrane exchange process that lead to the loss of sperm fertility.

It is not yet clear what is the mechanism of damage to sperm membranes in the process of preserving sperm by refrigeration.

Under these conditions, artificial inseminations as methods of reproduction allow to increase the selection intensity of the production rams and implicitly to increase the selection efficiency.

Of particular importance in this biotechnology is the methods of preserving and diluting ram semen in order to ensure proper fertility and birth. For these reasons, one of the most current perspectives is to develop new environments for preserving ram semen by refrigeration and introducing into their structure as an additional component of various biologically active antioxidants and cryoprotectants.

MATERIALS AND METHODS

The research took place during the breeding season.

The objective of these researches was to determine and statistically research the data on ram semen from the Moldovan Karakul breed. The biological material used was represented by 5 rams aged 3-4 years.
The criteria needed in the producers' choices were age and sexual behavior. The semen was harvested on sheep in heat or in anesthesia with the help of the artificial vagina due to the fact that this method of harvesting is fast and simple and the semen is superior both in terms of quality and quantity. After harvesting, the quantitative and qualitative parameters of the sperm were analyzed.
Macroscopic analysis of the appearance and volume of semen or determined immediately after collection in the graduated container attached to the artificial vagina.
After being followed by the microscopic ones regarding the mobility, concentration, speed of sperm advance (VAP-total speed, VSL-speed of sperm with rectilinear movements, VCL-speed of sperm with curvilinear movements) which were assessed using CEROS computer system.
Ejaculates with a mobility of more than 70% and a sperm concentration of more than 2 billion/ml were allowed for processing.
The ejaculates allowed for processing were diluted with the SȚJ medium. In the composition of which as an additional component was introduced the biologically active preparation LB/MP obtained from yeast yeasts from brewing by the Institute of Microbiology and Biotechnology of the Academy of Sciences of the Republic of Moldova.
The LB/MP preparation was introduced into the SȚJ environment component in the concentration range from 0.1% to 1%. After dilution and sperm mobility testing, the experimental batches were introduced into the cold room at +2-+4ºC.
Periodic determination of sperm motility at 24-hours intervals during storage of ram semen at refrigeration temperature in order to ascertain any differences between sperm motility and to establish the optimal storage period by refrigeration until the minimum mobility required for inoculation is reached.
Statistical parameters were calculated using the ISAS computer program.

RESULTS AND DISCUSSIONS

The study of the conservation of ram's semen by refrigeration, as well as the analysis of their qualitative parameters, the conservation techniques at refrigeration temperatures being the most used in these species have constituted and constitute the research directions in this field.
The success of sperm storage therefore depends on diluting the sperm with an environment containing substances that protect the sperm against the stress associated with lowering the temperature. The addition of different proportions of biologically active substances to a simple diluent, easy to prepare but which has also had a good effect on maintaining sperm motility over time is the main reason for perfecting the protocol for preserving sperm by refrigeration.
To establish the influence of the biologically active preparation on mobility
Experimental research on sperm was performed on ram semen diluted and supplemented with the experimental preparation LB/MP. The freshly sampled semen was diluted with the SȚJ diluent and supplemented with the preparation in various amounts from 0.1 to 1.0%, a total of 10 experimental batches. All batches, both experimental and control were subjected to storage at temperatures of +2-+4°C over a period of 120 hours. During storage, tests were performed every 24 hours to determine sperm motility.
Data on the mobility of sperm processed and stored at +2-+4°C for 120 hours are presented in Table 1.

Table 1. Mobility of ram sperm stored in time at +2-+4°C, %

Parameters		Witness (SŢJ)	LB/MP (%)									
			0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
fresh semen	Mobile, %	87.8 ±0.7	87.8 ±1.0	88.2 ±0.7	88.8 ±1.7	90.2 ±0.7	91.0 ±1.1	91.8 ±0.8*	92.5 ±1.3*	92.0 ±0.9*	91.3 ±0.6*	90.8 ±0.7*
	Progres, %	45.2 ±4.8	50.3 ±3.2	51.2 ±1.4	50.5 ±1.6	42.8 ±2.6	46.5 ±3.6	51.8 ±2.9	51.8 ±2.2	48.0 ±2.2	51.3 ±6.3	46.4 ±3.5
24 h	Mobile, %	83.8 ±2.0	83.8 ±2.0	87.6 ±1.4	86.5 ±1.3	88.8 ±1.7	90.5 ±1.2*	91.0 ±1.1*	91.8 ±1.1*	88.4 ±1.3	88.5 ±1.7	85.6 ±2.2
	progresive, %	36.8 ±2.1	33.0 ±3.9	42.2 ±4.1	38.0 ±6.6	46.2 ±4.4	40.8 ±3.8	47.0 ±3.9	45.8 ±5.3	39.6 ±3.9	37.3 ±5.6	39.6 ±5.6
48 h	mobile, %	81.2 ±1.0	82.0 ±1.4	84.2 ±1.0	85.3 ±1.2	86.6 ±0.7*	86.8 ±0.9*	86.8 ±1.2*	88.5 ±1.3*	87.0 ±1.1*	85.5 ±1.3	83.2 ±1.4
	progresive, %	38.6 ±5.2	39.8 ±5.9	39.8 ±4.4	37.8 ±4.0	34.8 ±1.7	33.0 ±2.2	43.6 ±4.6	42.8 ±3.1	38.6 ±3.8	45.8 ±5.3	40.0 ±5.5
72 h	mobile, %	71.2 ±3.9	78.5 ±1.7	74.0 ±4.5	80.3 ±0.9	77.8 ±4.2	82.5 ±1.0*	77.2 ±5.6	82.5 ±1.0*	78.2 ±3.1	79.0 ±1.6	75.0 ±3.7
	progresive, %	35.2 ±3.9	36.3 ±5.8	32.0 ±5.9	38.0 ±4.4	37.0 ±7.2	29.5 ±3.7	38.8 ±9.4	36.5 ±7.3	33.6 ±6.6	42.8 ±5.8	30.0 ±6.4
96 h	mobile, %	63.0 ±2.5	65.3 ±1.8	69.3 ±3.9	70.5 ±3.9	71.0 ±3.8	72.8 ±3.6	75.3 ±4.9	76.3 ±3.2*	77.0 ±3.5*	74.0 ±3.3	71.0 ±1.8
	progresive, %	26.0 ±4.1	24.8 ±3.3	28.8 ±4.8	24.8 ±3.9	33.0 ±8.4	26.8 ±4.8	33.0 ±5.4	32.8 ±7.5	38.5 ±4.9	30.8 ±5.9	31.8 ±6.7
120 h	mobile, %	58.3 ±2.0	57.3 ±2.7	61.8 ±5.3	62.5 ±4.9	63.0 ±5.1	63.0 ±5.3	66.3 ±5.8	67.5 ±4.6	68.0 ±5.8	65.0 ±5.4	62.3 ±4.8
	progresive, %	12.8 ±1.1	14.3 ±2.9	15.5 ±5.3	15.5 ±5.6	19.0 ±4.8	17.0 ±4.7	18.5 ±6.0	22.3 ±7.6	25.3 ±7.7	17.8 ±3.0	14.8 ±2.6

*P≤0.05

Research has shown that the LB / MP preparation produced at the Institute of Microbiology and Biotechnology of the Academy of Sciences of the Republic of Moldova introduced as an additional component in the environment of SŢJ in a concentration of 0.1% to 1.0% is not toxic to sperm in the range of the studied concentrations.

The data in Table 1 show that all the lots studied provided satisfactory values for the kinetic parameters, after 120 hours of storage. Sperm mobility was maintained at 68.0 ± 5.8% in the batch where the concentration of the LB / MP preparation introduced as an additional component in the TSJ medium by 0.8%.

Sperm mobility in the batch where the concentration of the LB/MP preparation introduced as an additional component in the TSJ medium in the amount of 0.7 (76.3 ± 3.2) and 0.8% (77.0 ± 3.5), after 96 hours of conservation showed positive values, statistically significant (P≤0.05) compared to the control group.

In batches with diluent supplemented with LB/MP in the amount of 0.5 to 1.0%, the kinetic parameters are maintained at a satisfactory level for a period of up to 6 days at a temperature of +4 degrees.

Further research determined the rate of straight line sperm (VSL), average speed (VAP), and curbolinal speed (VCL).

Data on the results of sperm movement speed in ram semen depending on the concentration of LB/MP introduced as an additional component in the STJ baseline during a shelf life of 120 hours are presented in Table 2.

The analysis of the results presented in Table 2 demonstrates that the speed of sperm advance is not significantly reduced in the experimental groups compared to the control group. Statistically significant differences were found between the diluent variant with the concentration of the preparation LB/MP of 0.8% (78.7 ± 6.2 μm/s,) and the control group after 5 days of keeping the semen diluted at temperatures of +4°C (P 0.05)

Table 2. Speed of sperm advance of ram, µm/s

Parameters		Witness (ȘȚJ)	LB/MP (%)									
			0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1
fresh semen	VAP	132.9 ±5.9	135.3 ±3.2	135.7 ±6.4	139.5 ±5.5	118.5 ±6.8	125.4 ±6.9	133.1 ±6.7	136.6 ±2.7	130.9 ±2.8	133.3 ±4.1	126.4 ±5.3
	VSL	109.9 ±6.7	113.8 ±4.0	114.0 ±5.2	117.1 ±5.1	94.6 ±5.3	103.8 ±5.9	110.4 ±5.6	112.1 ±3.9	106.6 ±2.5	112.0 ±5.7	106.4 ±7.6
	VCL	192.8 ±9.4	190.7 ±1.9	190.3 ±11.5	203.4 ±5.2	179.2 ±14.9	184.0 ±13.3	191.0 ±10.0	205.4 ±7.2	196.6 ±7.6	193.8 ±6.3	186.1 ±8.7
24 h	VAP	106.9 ±6.5	105.7 ±5.2	101.7 ±4.3	109.3 ±7.2	115.2 ±6.6	111.0 ±1.5	113.0 ±3.3	111.3 ±5.2	102.9 ±6.0	98.6 ±5.1	98.7 ±5.6
	VSL	84.3 ±6.5	78.1 ±2.4	80.5 ±5.3	83.8 ±7.5	92.0 ±6.7	85.0 ±3.0	89.2 ±3.2	89.2 ±5.8	80.4 ±3.9	76.8 ±5.2	80.0 ±5.7
	VCL	172.4 ±9.1	178.8 ±10.4	168.4 ±7.2	180.2 ±12.7	183.5 ±11.4	185.1 ±5.0	183.6 ±9.9	179.2 ±11.0	167.0 ±13.3	162.0 ±11.6	156.7 ±9.0
48 h	VAP	104.6 ±5.7	108.9 ±2.7	102.6 ±3.7	100.4 ±4.9	96.7 ±7.1	100.1 ±5.5	108.3 ±4.5	110.0 ±6.0	99.9 ±5.1	113.3 ±5.2	106.9 ±6.9
	VSL	85.7 ±7.2	86.2 ±4.6	79.7 ±3.9	80.1 ±5.2	71.8 ±1.9	75.9 ±4.6	83.2 ±4.3	82.4 ±4.2	78.5 ±4.2	91.2 ±6.5	86.0 ±6.8
	VCL	166.9 ±7.9	177.1 ±4.4	165.7 ±8.1	167.0 ±7.4	150.3 ±7.6	169.4 ±10.1	156.3 ±8.9	168.8 ±10.1	159.2 ±8.5	172.6 ±6.6	171.4 ±10.8
72 h	VAP	100.5 ±9.7	102.0 ±7.3	88.9 ±6.9	98.4 ±4.5	94.1 ±7.8	92.0 ±3.2	94.3 ±12.0	99.6 ±8.3	85.7 ±4.3	98.6 ±8.4	85.3 ±6.2
	VSL	82.3 ±9.5	83.2 ±7.9	71.0 ±6.8	80.4 ±5.9	77.2 ±7.9	69.7 ±3.7	81.6 ±9.8	78.8 ±9.3	68.3 ±4.6	81.9 ±9.1	67.8 ±6.7
	VCL	160.1 ±9.8	166.4 ±8.3	146.2 ±9.5	158.1 ±3.6	152.9 ±11.1	146.9 ±9.2	156.9 ±15.5	161.3 ±7.2	138.5 ±6.1	159.0 ±7.4	132.5 ±6.6
96 h	VAP	80.7 ±4.6	85.8 ±5.8	85.9 ±2.7	82.7 ±7.0	87.7 ±8.0	86.8 ±4.9	93.9 ±6.8	90.3 ±5.9	97.8 ±5.2	93.3 ±6.5	94.7 ±7.8
	VSL	57.9 ±4.1	67.8 ±5.3	65.5 ±3.1	66.4 ±5.8	73.0 ±8.1	67.0 ±6.0	76.3 ±9.1	71.5 ±8.1	78.7 ±6.2*	74.6 ±9.1	76.4 ±9.7
	VCL	145.1 ±8.1	147.6 ±6.2	150.5 ±2.5	144.6 ±11.8	142.7 ±10.3	155.0 ±8.0	156.8 ±5.1	155.1 ±5.1	158.9 ±4.8	154.1 ±4.8	158.8 ±5.0
120 h	VAP	73.1 ±2.0	76.1 ±1.5	72.7 ±7.9	74.6 ±6.7	79.2 ±6.2	74.1 ±4.8	76.7 ±5.4	76.4 ±6.9	80.3 ±9.4	72.4 ±3.6	71.2 ±2.7
	VSL	54.8 ±0.6	55.4 ±3.9	57.0 ±5.2	52.0 ±3.6	58.5 ±5.2	56.1 ±5.6	56.6 ±6.6	58.2 ±7.5	64.4 ±9.0	52.9 ±3.4	52.8 ±2.7
	VCL	137.6 ±6.1	136.0 ±6.7	125.0 ±14.6	131.2 ±12.1	135.4 ±11.9	132.0 ±6.4	133.0 ±7.6	132.0 ±11.3	136.7 ±12.9	126.3 ±6.9	125.0 ±6.0

*P≤0.05

In conclusion, the diluent supplemented with the LB/MP preparation in the proportions from 0.1 to 1.0%, experienced in our research showed positive results during the longer storage of semen.

Determining the morphological integrity of semen is an important parameter for assessing semen quality. More often, abnormalities are detected in the tail of sperm, the base of the head and neck. A significant number of pathological sperm should certainly be seen as a sign of impotence.

The research conducted aimed to determine how the LB/MP preparation, introduced as an additional component in the ȘȚJ environment. Influences the morphological parameters of ram semen in the storage period of semen at refrigerator temperatures.

For this we identified possible differences in morphological parameters, depending on the percentage of LB/MP added to the diluent ȘȚJ (Table 3).

Table 3. Abnormalities of ram semen

	parameters	Witness (STJ)	STJ + LB/MP, %				
			0.2	0.4	0.6	0.8	1.0
fresh semen	Macrocephaly	0.7±0.3	0.7±0.7	1.0±0.6	0.7±0.3	0.3±0.3	0.3±0.3
	Microcephaly	1.0±0.6	0.7±0.3	0.3±0.3	0.3±0.3	0.7±0.3	0.7±0.3
	Broken neck	6.3±0.9	6.0±0.6	6.0±0.6	5.0±0.6	4.7±0.3	5.0±0.6
	Double head	-	-	-	-	-	-
	Twisted tail	5.3±0.7	5.3±0.3	4.3±0.9	4.0±0.6	4.7±0.9	4.3±0.3
	Headless	4.0±0.6	4.0±0.6	3.3±0.3	3.0±0.6	3.0±0.0	3.7±0.3
	No tail	4.7±0.3	4.0±0.6	4.3±0.7	4.7±1.2	4.3±0.3	4.3±0.7
	Total-Total, %	**22-11**	**20.7-10.4**	**19.2-9.6**	**17.7-8.9**	**17.7-8.9**	**18.3-9.2**
24 h	Macrocephaly	1.0±0.6	1.0±0.6	1.0±0.6	0.7±0.3	0.7±0.3	1.0±0.6
	Microcephaly	1.0±0.6	0.7±0.7	1.0±0.6	1.0±0.6	1.0±0.6	1.0±0.6
	Broken neck	6.7±0.7	6.3±0.3	6.0±0.6	6.0±0.6	6.0±0.6	6.3±0.3
	Double head	-	-	-	-	0.3±0.3	0.3±0.3
	Twisted tail	6.0±0.6	6.0±0.6	5.3±0.9	5.0±0.6	5.0±0.6	5.3±0.3
	Headless	5.7±0.7	5.3±0.3	5.3±0.3	5.0±0.6	5.3±0.9	5.3±0.3
	No tail	5.3±0.9	5.3±0.9	5.3±0.3	5.7±0.7	6.3±0.3	6.3±0.3
	Total-Total, %	**25.7-12.9**	**24.6-12.3**	**23.9-11.9**	**23.4-11.7**	**24.6-12.3**	**25.5-12.8**
48 h	Macrocephaly	1.3±0.3	1.0±0.0	1.0±0.6	0.7±0.3	1.3±0.9	1.3±0.7
	Microcephaly	1.0±0.6	1.3±0.3	0.7±0.3	0.7±0.3	0.7±0.3	1.0±0.6
	Broken neck	7.7±0.9	8.0±0.6	7.7±0.3	7.3±0.9	7.3±0.3	7.7±0.7
	Double head	-	-	-	0.3±0.3	-	-
	Twisted tail	6.7±1.2	6.7±0.9	6.7±1.2	7.0±0.6	6.3±1.2	7.0±0.6
	Headless	7.7±0.9	7.7±0.7	7.3±0.3	7.0±1.5	7.3±1.2	7.7±1.2
	No tail	7.7±2.8	7.7±0.9	8.3±0.3	8.3±1.5	7.7±0.3	8.0±1.0
	Total-Total, %	**32.1-16.05**	**32.4-16.2**	**31.7-15.9**	**31.3-15.7**	**30.6-15.3**	**32.7-16.4**
72 h	Macrocephaly	1.3±0.7	1.0±0.0	1.0±0.0	1.0±0.6	1.0±0.0	1.3±0.3
	Microcephaly	1.0±0.6	0.7±0.3	1.0±0.6	0.7±0.3	1.0±0.6	1.0±0.6
	Broken neck	8.0±1.5	8.0±2.1	7.7±2.0	7.3±0.9	7.3±0.7	7.7±0.9
	Double head	-	--	-	-	-	-
	Twisted tail	7.0±0.6	7.0±1.2	7.0±0.0	6.7±0.3	6.7±0.9	7.0±0.6
	Headless	8.0±0.6	7.7±0.7	7.0±0.6	6.3±0.9	6.3±0.3	6.3±0.3
	No tail	8.0±0.6	8.0±0.6	8.3±1.5	8.3±1.2	8.0±1.5	8.0±1.0
	Total-Total, %	**33.3-16.7**	**32.4-16.2**	**32-16**	**30.3-15.2**	**30.3-15.2**	**31.3-15.7**
96 h	Macrocephaly	1.7±0.3	1.3±0.3	1.0±0.0	1.0±0.6	1.0±0.6	0.7±0.3
	Microcephaly	1.0±0.6	0.7±0.3	0.7±0.3	0.7±0.3	0.7±0.3	0.7±0.3
	Broken neck	8.3±0.3	8.3±0.9	8.7±0.7	8.0±0.0	7.0±1.0	7.7±0.9
	Double head	-	-	0.3±0.3	-	-	-
	Twisted tail	7.7±0.3	8.0±0.0	7.3±0.3	7.7±0.3	7.3±0.3	7.3±0.9
	Headless	8.0±1.2	8.0±0.6	7.7±0.9	7.3±0.3	6.7±0.9	6.7±0.3
	No tail	8.7±0.9	8.3±1.3	9.0±1.5	8.7±1.5	8.7±0.9	8.3±0.3
	Total-Total, %	**35.4-17.7**	**34.6-17.3**	**34.7-17.4**	**33.4-16.7**	**31.4-15.7**	**31.4-15.7**
120 h	Macrocephaly	2.0±0.6	1.7±0.3	1.7±0.3	1.3±0.7	1.3±0.3	1.7±0.7
	Microcephaly	1.0±0.0	1.0±0.0	1.0±0.0	1.3±0.3	1.0±0.0	1.3±0.3
	Broken neck	8.7±0.3	9.0±0.6	8.7±0.3	8.7±0.9	7.7±0.9	8.3±0.9
	Double head	0.3±0.3	0.0±0.0	0.0±0.0	0.0±0.0	0.3±0.3	0.0±0.0
	Twisted tail	8.3±0.3	8.0±0.6	7.7±0.9	8.0±0.6	7.7±0.3	8.0±1.2
	Headless	8.3±1.3	8.3±1.2	8.3±0.3	8.3±0.7	7.3±0.3	7.7±0.7
	No tail	9.0±0.0	8.7±0.3	8.7±0.9	9.0±1.5	8.3±0.3	8.7±0.9
	Total-Total, %	**37.6-18.8**	**36.7-18.4**	**36.1-18**	**36.6-18.3**	**33.6-16.8**	**35.7-17.9**

The data in the table show that the highest average value of morphologically abnormal spermatozoa, at the beginning of the experiment, was in the control group in which

the ram semen was diluted with SȚJ medium. When the sperm were diluted with SȚJ medium, in which the LB/MP preparation was introduced as an additional component, at a concentration of 0.8%, the mean value of the abnormal shape indicators was the lowest and was 8.9%. After 120 hours of storage, ram semen diluted and stored at +2-+4°C, the morphological parameters of this experiment indicate that the average value of sperm with abnormality is still high in the control - 18.8%, while in the seminal material with SȚJ medium diluted with the addition of 0.8% of the LB/MP preparation, the morphological parameters corresponded to the standard, the values being the best - 16.8%.

The effect of the LB/MP preparation introduced as an additional component in the SȚJ environment on the condition of the acrosome of ram sperm during storage of sperm at refrigeration temperatures was determined (Table 4).

Table 4. Spermatozoa with damaged acrosome in ram semen stored at temperature +2-+4°C

Specification		Sp/pr	24 h	48 h	72 h	96 h	120 h
Witness (SȚJ)		4.0±0.8	9.0±0.7	24.5±3.5	31.0±3.5	45.3±3.7	60.8±5.3
LB/MP, %	0.1	4.0±1.0	8.2±0.9	14.2±3.7	27.0±4.0	41.7±4.9	55.2±3.5
	0.2	4.0±0.7	8.1±0.5	14.5±2.9	28.3±3.5	42.4±3.7	56.5±4.9
	0.3	3.3±0.9	7.5±0.3	13.0±3.3	24.7±4.2	41.2±4.6	52.3±4.6
	0.4	3.5±0.9	7.5±0.6	11.9±2.0	25.8±3.5	40.9±3.6	56.1±4.4
	0.5	3.0±0.9	7.3±0.3	11.0±2.8	22.8±4.4	38.3±3.6	52.7±4.4
	0.6	3.1±0.5	6.8±0.3	10.5±2.0*	24.9±3.7	39.8±3.2	55.1±4.6
	0.7	2.7±1.0	6.5±0.3*	10.3±3.3	21.8±4.1	37.0±3.8	51.7±4.4
	0.8	2.6±0.7	6.6±0.2*	10.3±2.4*	24.5±3.9	36.4±2.2	52.4±3.4
	0.9	2.8±0.7	6.5±0.6	10.5±3.8	23.2±3.4	30.3±3.9	52.2±4.4
	1.0	3.1±0.6	7.6±0.6	11.9±2.3	25.8±3.5	40.1±3.4	56.4±4.8

*P≤0.05

The data presented in the table show that at the initial dilution, the lowest percentage of sperm with damaged acrosome was in the sperm with SȚJ medium, with the addition of 0.7 to 0.9% of the LB/MP preparation, which constituted 2.6-2.8% compared to the control group, where the percentage of damaged acrosomes was 4.0%.

The most efficient variant was experienced in sowing sheep. For this purpose, the ejaculates allowed for processing were diluted with the SȚJ medium in the composition of which as an additional component the LB/MP preparation was introduced in a concentration of 8%. After dilution the sperm was exposed to temperatures of 2-4 degrees for 120 hours, after which the refrigerated semen was used for artificial insemination of sheep. Experimental data on sheep sowing are presented in Table 5.

Table 5. The results of artificial insemination of sheep

Breed	They were Sown (heads)	They didn't repeat		The heat gave birth	
		heads	%	heads	%
Karakul	74	41	53.4	36	48.6

The detection of sheep in heat was carried out with the help of test rams. The first time the sheep were sown after being detected, and the second sowing over 10-12 hours after the first sowing. The results of artificial insemination showed that out of 74 sheep artificially seeded with refrigerated semen and stored for 120 hours, they gave birth to 36 heads or 48.6%.

CONCLUSIONS

The LB/MP preparation introduced as an additional component in commercial dilution media is not toxic for ram sperm in the range of concentrations studied (0.1-1.0%).

After 6 days of storage of the temperature +2-+4°C the best results were obtained when the concentration of the LB/MP preparation introduced in the dilution media was 0.6-0.8%: - mobility 67.5-68.0%, permissible mobility for artificial insemination; VSL - 58.2-64.4 μm/s, VCL - 132.0-136.0 μm/s;

The percentage of abnormal sperm was 33.6-36.6% in the experimental groups compared to the control group in which the number of sperm was 37.6%.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

INVESTIGATION SOME PLACENTAL TRAITS IN GOATS OF BULGARIAN WHITE DAIRY BREED AND THEIR CROSS-BREEDS

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Abstract

The aim of our study was to investigate the relationship between some placental parameters such as Placental weight (PW), Cotyledon number (CN), Placental efficiency (PE) and Cotyledon density (CD), as well as the Litter weight (LW) with the genotype of goats, type of birth and the gender of kids. The study was conducted in the goat farm of the RIMSA, Troyan, Bulgaria. The study involved 94 goats of 53 Bulgarian White Dairy breed (BWD) and its crossbreeds with Anglo-Nubian (AN)21 and Togenburg (TG)20. The results showed that PE in goats BWD was significantly higher compared to BWD x AN, BWD x T. There was a strong downward relationship between PW and PE. A significant downward relationship between PW and CD was found in all three genotypes. The PW of twins is higher than that singles ($p<0.01$). The total CN in twins is higher than in singles ($p<0.01$). A significant positive relationship was found between PW and LW in all studied genotypes and was highest in BWD x AN ($p>0.05$). A significant positive relationship was found between PW and the total CN in BWD.

Key words*: breed, goats, placental efficiency, placental weight.*

INTRODUCTION

Through the placenta, the fetus receives nutrients and oxygen from the mother and removes unnecessary substances from its metabolism. The development of the placenta is influenced by various factors (maternal nutrition, breed, parity of goat, litter size, sex, etc.). The postnatal survival of the newborn also indirectly depends on these factors.

Size at birth is critical in determining life expectancy. It affects not only neonatal viability but also adult rates of morbidity and mortality (Fowden & Forhead, 2009).

Sen & Önder (2016) describe the characteristics of the placenta as one of the most important indicators of fetal growth and development that affect the vitality of kids.

According to Mellor & Stafford (2004), the factors that determine the survival and mortality of the newborn are placental insufficiency and hypothermia. Nutrient delivery to the fetus depends on a number of critical factors, which include placental growth and development, uteroplacental circulation, nutrient availability, placental metabolism, and transport capacity (Dunlap et al., 2015).

The size of the cotyledons is known to be crucial for the prenatal and postnatal survival of kids. The number of cotyledons varies between breeds, as within the breed, and is influenced by litter size, sex and etc (Alexander, 1964).

In hair goats, Ozyurek (2019) studies the relationship between kids vitality and placental characteristics. In their study it was found that placental efficiency was affected by the vitality of kids ($p<0.05$) and observed that there was no x large cotyledon (> 51 mm diameter) in the dead kids.

Some authors (Dwyer et al., 2005; Konyalı et al., 2007; Alkass et al., 2013) mention that smaller placentas are more effective than large ones because large ones need more nutrients.

The Bulgarian white dairy goat breed is the main dairy goat breed that is bred in Bulgaria. There are studies on placental parameters in different breeds of goats, but in the breed Bulgarian White Dairy there are none. For this reason, the aim of our study was to examine some placental parameters such as placental weight, cotyledon number, placental efficiency and cotyledon density, as well as litter weight and their relationship to the goat breed

(genotype), type of birth and sex of kids in the Bulgarian white dairy breed of goats and it's crosses with the Anglo-Nubian and the Togenburg breeds.

MATERIALS AND METHODS

The study was conducted in the goat farm of the Research Institute on Mountain Stockbreeding and Agriculture in the town of Troyan, Bulgaria. The facility is located at an altitude of 380 m (42°53'39"N/24°42'57"E).

The study involved 94 goats of 53 Bulgarian White Dairy breed (BWD) and its crossbreeds with Anglo-Nubian (AN)21 and Togenburg (TG)20. There were 53 singleton (31 male and 22 female) and 41 twin pregnancies.

All goats were housed and cared for under the same conditions. During the winter period animals were kept in a barn and fed with a ration containing of 2 kg hay, and 0.8 kg concentrated fodder per head. There was free access to water and salt. In the spring months (May-November) goats were grazing.

Goats were vaccinated against enterotoxemia, treated for parasites, and given vitamins A, D, and E (Vialiton, Biovet).

Kidding of goats took place in February and March. Before kidding goats were separated in individual pins and were under surveillance. Placentas (chorioalanantois and related fetal cotyledons) were collected immediately after natural delivery and weighed fresh in digital scales.

The kids were weighed right after birth. When twins were born the weight of each placenta was summed up.

The Cotyledon number (CN) from each delivered placenta was counted and recorded. Cotyledons were classified by size according to Konyali et al. (2007). Cotyledon number (< 10 mm diameter); Cotyledon number (between 10 and 30 mm diameter); Cotyledon number (≥ 3 mm diameter).

Placental efficiency was defined as the ratio of total kid birth weight (g) to Placental weight, (g) (Molteni et al., 1978).

Cotyledon density was defined as the number of cotyledons per gram of Placental weight (Ocak et al., 2013).

One-way ANOVA was used for statistical comparison. Relationships between the placental traits were determined with a Pearson correlation analysis.

RESULTS AND DISCUSSIONS

The placental parameters (placental weight, cotyledon number, placental efficiency and cotyledon density) and the litter weight according to the goat genotype and the type of birth are presented in Table 1.

Table 1. Placental parameters and total weight of the born kids according to genotype and type of birth

	Litter weight, g LW x S_x	Placental weight, g PW x S_x	Cotyledon number CN				Placental efficiency PE x S_x	Cotyledon density CD x S_x
			total x S_x	≤1 x S_x	1-2 x S_x	≥3 x S_x		
BWD	a*5749.62± 259.78	637.17± 29.83	84.92± 3.62	8.62± 1.11	68.10± 3.53	7.62± 0.89	9.43± 0.36 a** c*	0.14± 0.01
BWD x AN	a*4771.43± 394.37	628.57± 52.83	86.52± 5.76	11.52± 2.00	67.48± 5.89	7.52± 2.01	7.84± 0.41 a**	0.15± 0.01
BWD x T	4820.00± 371.44	627.00± 43.20	81.25± 4.67	6.16± 1.17	68.30± 4.47	7.05± 1.17	7.89± 0.44 c*	0.14± 0.02
Total singleton	b**3961.32± 97.96	b**526.42± 22.98	b**73.36± 3.11	9.67± 1.12	58.33± 3.05	5.75± 0.89	8.03± 0.30	0.15± 0.02
Singleton male	4109.68± 143.80	522.26± 30.73	73.97± 4.11	8.93± 1.50	59.80± 4.00	5.94± 1.35	8.39± 0.40	0.15± 0.01
Singleton female	3765.91± 107.98	527.27± 35.99	73.09± 4.70	10.68± 1.71	56.55± 4.74	5.86± 1.05	7.66± 0.43	0.15± 0.01
Twins	b**7106.83± 206.08	b**770.98± 30.22	b**98.90± 3.20	3.20± 1.20	80.27± 4.79	9.71± 1.06	9.67± 0.40	0.13± 0.01

Note: a-BWD/BWD x AN; b-total singleton/ twins; c-BWD/BWD x T ;*p<0.05,**p<0.01

Although the litter weight of the Bulgarian White Dairy breed goats was significantly higher than that of the Bulgarian White Dairy x Anglo-Nubian with a difference of 978 grams, the placental weight of the three genotypes we studied was almost the same. Placental effectiveness in the Bulgarian White Dairy breed is significantly the highest. According to Ocak & Önder (2011), when studying the effects of the breed it is important to identify the effects of the maternal and fetal genotype as they have an effect on gestational period and birth weight. Alkass et al. (2013) studied two breeds of goats bred in Iraq and found that the effect of the breed is not a reliable source of variation in all studied placental traits. The studies of Ocak et al. (2009) in different breeds and (genotypes) of sheep are similar. Jawasreh et al. (2009) and Oramari et al. (2011) report that the litter weight is strongly influenced by placental weight in Awasi and Karadi lambs.

We found that logically the litter weight increases ($p<0.01$) with an increase in the number of offspring from one to two by 3145 g. The placental weight of the twins was significantly higher than that of the single ones ($p<0.01$). The total number of cotyledons in twins was significantly higher than in singles ($p<0.01$). This could be explained by the statement of Dwyer et al. (2005), that the uterus of sheep with a smaller number of caruncles would not be able to carry more than one fetus. Ocak et al. (2015) define the cotyledons of twins as larger and heavier than those of singles, which would explain the higher effectiveness of their placenta. Our study showed that the placental effectiveness of twins is higher than that of singles. These results are in agreement with Ocak et al. (2015), according to whom different-sex twins have better placental efficiency than same-sex twins in Damascus goats. Ozyurek & Türkyilmaz (2020) found that in Morkaraman sheep the type of birth has a significant effect on the birth weight, placental weight, and the cotyledon number. Male singles were heavier than female singles, which is in line with our previous studies (Hristova et al., 2013; Stoycheva, 2014) in the same genotypes as found by Ozyurek (2019) in hair goats and Ocak et al. (2009) in sheep. Despite the observed difference in weight according to the gender, the weight of their placentas was almost the same.

Contrary to the findings of Ocak et al. (2015), we observed lower placental effectiveness in the placentas of male singles than females ($p>0.05$). No significant difference was found in the total number of cotyledons between the genotypes studied by us, as well as between males and females. The density of cotyledons for all groups studied by us was almost the same. Table 2 presents the correlations between placental parameters according to the genotype of the goats.

Table 2. Pearson correlation coefficient of placental parameters by breed (genotype)

	LW	CN	PE	CD
BWD				
PW	0.653**	0.576**	-0.446**	-0.554**
LW		0.610**	0.331*	-0.217
CN			-0.059	0.292*
PE				0.387**
CD				
BWD x AN				
PW	0.730	0.413	-0.358	-0.568**
LW		0.120	0.336	-0.581**
CN			-0.477*	0.393
PE				-0.047
CD				
BWD x T				
PW	0.643**	0.404	-0.361	-0.600**
LW		0.444*	0.449*	-0.288
CN			0.004	0.444*
PE				0.306
CD				

*p<0.05, **p<0.01

Significant positive relationship between placental weight and liter weight ($p<0.01$) was found in Bulgarian White Dairy goats ($r = 0.653$), Bulgarian White Dairy x Togenburg ($r = 0.643$) and the highest in Bulgarian White Dairy x Anglo- Nubian ($r = 0.730$, $p>0.05$), which is in line with that found by Konyali et al. (2007) in Turkish Saanen goats.

The weight of the fetus was related to the weight of the placenta, the two together formed the whole of the tissues of the mother and the fetus and thus the placenta was the factor determining the growth of the fetus (Konyalı et al., 2007; Ocak & Onder, 2011).

A significant positive relationship was found in Bulgarian White Dairy breed goats between placental weight and total number of cotyledons ($r = 0.586$, $p<0.01$).

We found a significant negative relationship between placental weight and cotyledon density ($r = -0.554$; -0.568; -0.600, $p<0.01$) in all three breeds (Bulgarian White Dairy, Bulgarian White Dairy x Anglo - Nubian, Bulgarian White Dairy x Togenburg), which is in agreement with Ocak et al. (2009) in sheep. Significant negative dependence was also found between liter weight and cotiledon density in Bulgarian White Dairy breed x Anglo-Nubian ($p<0.01$).

Table 3 presents the correlations of the placental parameters according to the gender and the type of birth.

Table 3. Pearson's correlation coefficient of placental parameters according to the gender and the type of birth

	LW	CN	PE	CD
Singles				
PW	0.479**	0.311*	-0.746**	-0.528**
LW		-0.140	0.165	-0.586**
CN			-0.441	0.560**
PE				0.193
CD				
Singles male				
PW	0.428*	0.373	-0.728**	-0.493**
LW		-0.207	0.257	-0.687**
CN			-0.554	0.532**
PE				-0.008
CD				
Singles female				
PW	0.700**	0.247	-0.836**	-0.598**
LW		-0.096	-0.292	-0.607**
CN			-0.381	0.554**
PE				0.482*
CD				
Twins				
PW	0.363*	0.312	-0.721**	-0.687**
LW		0.246	0.285	-0.124
CN			-0.244	0.265
PE				0.583**
CD				

*p<0.05; **p<0.01

Ocak et al. (2009) found that placental weight, placental efficiency, and cotyledon number were influenced by the type of birth of the lambs and not by their gender. We found a significant negative relationship between placental weight and cotyledon density in singles ($r = -0.528$, $p<0.01$), in females ($r = -0.598$, $p<0.01$), and in twins ($r = -0.687$, $p<0.01$).

Significant negative dependence between liter weight and cotyledon density was observed in all singles ($r = -0.586$, $p<0.01$), in males ($r = -0.687$, $p <0.01$) and in females ($r = -0.607$, $p<0.01$). Our study also showed that there was a significant positive relationship between cotyledon number and cotyledon density ($r = 0.560$, $p<0.01$) in singles, in single males ($r = 0.532$, $p<0.01$) and in single females ($r = 0.554$, $p<0.01$).

In male singles, we found a significant negative relationship between both cotyledon number

and placental efficiency ($r = -0.554$, $p<0.01$). In female singles, we found a strong positive relationship between placental weight and liter weight ($r = 0.700$, $p<0.01$). In twins, we found a significant positive relationship between placental weight and cotyledon density ($r = 0.583$, $p<0.01$).

A strong negative relationship between placental weight and placental effectiveness was found in singles ($r = -0.746$, $p<0.01$), in male singles ($r = -0.728$, $p<0.01$), in female singles ($r = -0.836$, $p<0.01$).) and in twins ($r = -0.721$, $p<0.01$), ie with the increasing weight of the placenta its effectiveness decreased. According to Wilson & Ford (2001), the placental efficiency determines how many grams of the fetus fall per 1 g of placenta, which means that the placental efficiency determines the capacity of the mother.

Our findings support the claims of many authors (Mesa et al., 2003; Dwyer et al., 2005; Ocak et al., 2009) that smaller placentas are more effective. According to the authors, this is due to lower nutrient expenditure.

CONCLUSIONS

The results of this study showed that the placental effectiveness in Bulgarian White Dairy breed goats is significantly higher compared to crosses Bulgarian White Dairy x Anglo-Nubian and Bulgarian White Dairy x Togenburg.

As the weight of the placenta increases, its effectiveness decreases. A strong negative relationship was found between placental weight and placental effectiveness ($p<0.01$) in single and twin as well as male and female kids.

A significant negative relationship was found between placental weight and cotyledon density ($p<0.01$) in all three genotypes (Bulgarian White Dairy breed, Bulgarian White Dairy x Anglo-Nubian, Bulgarian White Dairy x Togenburg).

The weight of the placenta of twins is higher than that of singles ($p<0.01$). The total number of cotyledons in twins is higher than in singles ($p<0.01$).

A significant positive relationship was found between placental weight and liter weight in all studied genotypes ($p<0.01$).

A significant positive relationship was found in Bulgarian White Dairy goats between placental weight and the total cotyledon number ($p<0.01$).

Further studies are required to investigate the relationship between the genotype and the placental parameters in Bulgarian White Dairy breed goats and its crossbreeds.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

DEVELOPMENT AND DIFFERENTIATION OF THE INTERMUSCULAR AND SUBMUCOSAL NERVE PLEXUS OF COW EMBRYOS LARGE INTESTINE

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Abstract

The article describes the development and differentiation of the intermuscular and submucosal nerve plexuses of the large intestine of the fetuses of cows from 50 days of age to birth. On days 50-70, the muscular membrane of the intestinal wall is built from bundles of smooth muscle cells, clearly divided into inner (annular) and outer (longitudinal) layers. Ganglia are laid between them, forming the intermuscular nerve plexus. The submucous nerve plexus differentiates later. The development of the nervous apparatus of the rectal wall is ahead of the development of similar structures of the colon and cecum. Enhanced growth and differentiation of nerve cells in fetuses is noted from 95-120 days. The first synapse-like contacts were observed in the intestinal ganglia at the age of 4.5 months of embryonic development. Enhanced differentiation and growth of nerve cells of the submucous nerve plexus is observed in fetuses of a cow at 6-7 months. By the end of the embryonic period and in early postnatal ontogenesis, connective tissue gradually overgrows the developing ganglion of the colon wall. Before the birth of the fetus, the nervous tissue of the colon wall forms a complex intramural nervous system, represented by four plexuses - intermuscular, submucosal, actually mucous and sub-serous. There are a large number of mature synaptic contacts. The study examines the stages of morphological changes in the development of intestinal ganglia from the aggregation of neural cells to the formation of a complex intramural nervous system in the walls of the colon.

Key words: *glia, glial processes, intermuscular nerve plexus, nerve fibers, neuroblasts, neurons, preganglionic fibers, submucosal nerve plexus, synaptic vesicles.*

INTRODUCTION

The study of the patterns of development of the digestive system, including the large intestine, is an important prerequisite for the development of a nutritional system, prevention and diagnosis of various diseases.

A significant number of studies devoted to the digestive system of animals touch upon general questions of the structure and patterns of growth of the gastrointestinal tract in ontogenesis. The morphology and histology of the small intestine has been studied in some detail (Bogolepov, 1994).

Analysis of the available sources shows that the large intestine in fetuses and in newborn calves (especially its immune and nervous systems) has not been sufficiently studied from the histological and cytochemical points of view. Therefore, in the course of the study, tasks were set and solved for the study of 1) the laying, formation and specialization of the nerve plexuses of the colon in fetuses and newborn calves; 2) the development and differentiation of the intermuscular and submucosal nerve plexuses of the large intestine of the fetuses of cows from 50 days of age to birth; 3) consideration of the stages of morphological changes in the development of intestinal ganglia from the aggregation of neural cells to the formation of a complex intramural nervous system in the walls of the colon (Sosunov, 1996).

MATERIALS AND METHODS

The material for the study was fetuses and newborn calves, their colon (its components: blind, colon, rectum). Pieces of tissue were taken from different parts of the colon: cranial, middle, and caudal. The material was fixed by a standard method for histological, histo-physiological and histochemical studies (Teltsov, 2002). Cytometry was performed by measuring the short and long diameters of the nucleus, the height and width of the cell

(Muller, 1992). The area of the nucleus and the cell was calculated and the nuclear-cytoplasmic ratio was derived. For the statistical analysis of all results, such indicators as the arithmetic mean, the arithmetic mean error, the correlation coefficient, and the error probability were used (Furness, 2000).

RESULTS AND DISCUSSIONS

At 3 months of age, the fetuses of cows, the nerve ganglia of the colon wall become more compact, the number of cellular nerve elements and fibers increases. The glial layers in the ganglia are well developed, and on the periphery of the ganglion there are flattened bodies and leaf-like processes of cells, which can be considered glioblasts. It should be noted the presence of desmosome-like contacts between neuroblasts and glial cells. On the periphery of the ganglion, there are small bundles and individual collagen fibers that limit it from the surrounding tissues. At this time, synaptic contacts appear with osmiophilic active zones and a few small light-colored synaptic vesicles. There are also large granular vesicles in the cell bodies and in the nerve processes. In the cytoplasm of neuroblasts, single tubules of the granular endoplasmic reticulum appear. In neuroglial cells, the content of tubules of the endoplasmic reticulum also increases. At this time, one can see how glial cells surround large bundles of nerve fibers.

Sections at this age reveal from 8 to 12 neuroblasts, single differentiating young neurocytes. The latter are polygonal and form one or two processes.

In fetuses of 90-120 days of age, in the nerve ganglia lying in the area of mesentery attachment, along with a large number of neuroblasts (10-20), multi-process young differentiating neurocytes are revealed (Figure 1).

In fetuses of cows 3-5 months with age, the number of mature cells with a large nucleus increases in the ganglia. At this time, neuro- and glioblasts can be clearly differentiated by the shape of the nucleus and body.

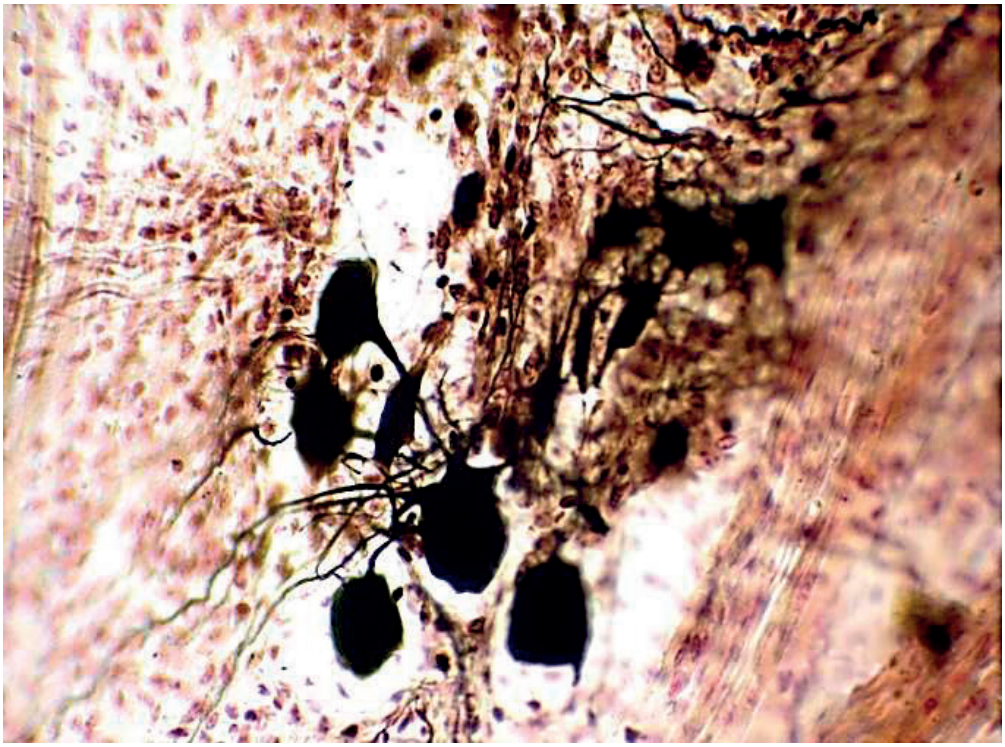

Figure 1. Section of the intermuscular nerve plexus of the colon wall of a 4-month-old fetus

Large nerve processes are located both in the center and periphery of the ganglion.

The loops of the intermuscular nerve plexus acquire a varied shape, the bundles of nerve fibers that form them become more powerful, the ganglia are located at the intersections and branching of the bundles, the neurons are at various stages of differentiation.

At 4-4.5 months of age, most of the neurocytes have a well-developed lamellar Golgi complex, with a large number of bordered vesicles near its cisterns. Large granular vesicles and small vesicles are also found in many cells. At the age of 4-4.5 months it is possible to isolate synapses in the intermuscular nerve plexus. In the early stages of their development, synapses look likedelicate buttons, dumbbells, loops that end on the bodies of nerve cells. In this case, near the nucleus, the thinnest branches of the preganglionic fibers. At the point of contact, a small depression is formed on the body of the neuron.

In fetuses of cows at the middle stage of development (from 5 to 7 months), the ganglia become even more dense, due to a decrease in the volume of free intercellular space. Many neuroblasts and young neurons are in contact with each other, forming simple and desmosome-like connections. This is due to the fact that the processes of glial cells, especially in the central parts of the forming ganglia, are still not well developed. The ganglia include type 1 Dogel cells. In close connection with these cells, neurons arise with two to four long, multiply branching processes that extend from different poles of the cell and leave the ganglion. Neurons with such processes in their morphological characteristics belong to type 2 Dogel cells. Dogel type 2 cells are pear-shaped, elongated, body contours are smooth, and processes are long.

The presence of long and short (receptor) dendrites is noted. Long dendrites extend from the poles, penetrating the ganglion and without branching, extend far beyond the ganglion. Short dendrites, shortly after leaving the cell body, repeatedly branching and form receptor endings either on the territory of the ganglion or outside it.

At the late stage of fetal development in cows (from 7 months to birth), the volume of the neuropil increases, and the number of synaptic contacts also increases. The active zones become longer, the number of small synaptic vesicles, characterized by a variety of shapes and sizes, increases significantly (Figure 2). In many differentiating neurons, processes with well-developed cytoplasmic organelles were observed.

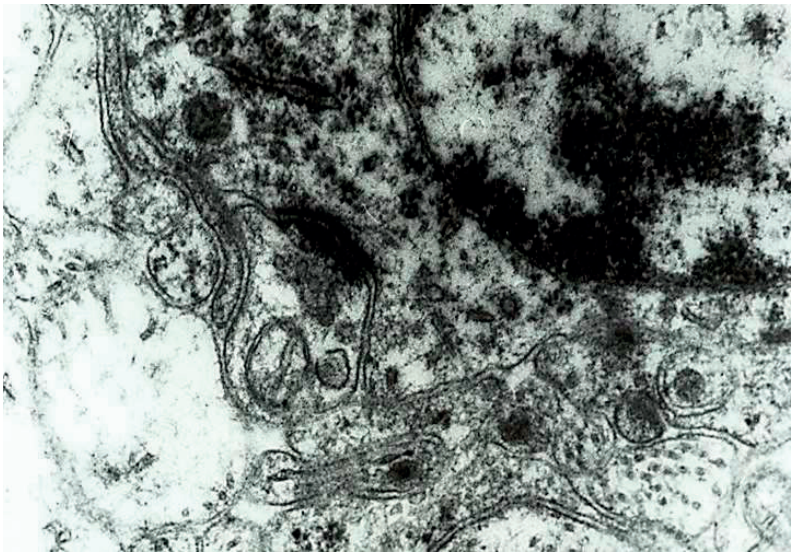

Figure 2. Ultrastructure of specialized interneuronal contact of the intermuscular nerve plexus of the rectal wall of a 4.5-month-old fetus. Uv. 30,000

Sometimes it was possible to observe a close arrangement of bundles of nerve processes directly near the cytolema of a smooth muscle cell. During this period, nerve fibers with varicose veins filled with large granular vesicles, mitochondria and single synaptic vesicles are found in the neuropil of the ganglion.

On drugs at this age, the number of differentiating neurocytes increases. The number of nucleoli in them decreases, but as a rule, the neurocyte still contains 2 or more nucleoli. At the late stage of fetal development, the nerve fibers and ganglia of the intermuscular nerve plexus form a large-looped network. There are significantly more differentiating nerve cells in the ganglia of the intermuscular nerve plexus than in the ganglia of the submucosal nerve plexus. The caudocranial gradient in the development of nerve tissue remains at this age. It can be traced when comparing the level of differentiation and the degree of innervation of the walls of the blind, colon and rectum. The most differentiated ganglia are the nerve plexuses of the rectum, less - the colon and blind. Before birth, already well-formed nerve plexuses are presented as mature cells, and neuroblasts and glioblasts, as well as a large number of nerve fibers. Nerve cells differ in the area of the perikaryon and especially clearly in the organization of nuclear chromatin. In the nerve ganglion, the walls of the rectum, cells and nerve fibers are located compactly, there is practically no free intercellular space. Poorly differentiated cells also lie in compact groups. Nerve and glial cells differ well both in chromatin density and in the organization of the cytoplasm. In these terms, the organization of the ganglionic neuropil becomes much more complicated. An increase in the number of their own processes in nerve cells and a weak development of glial membranes leads to the formation in the nerve ganglion, the walls of the rectum, cells and nerve fibers are located compactly is practically no free intercellular space. Poorly differentiated cells also lie in compact groups. Nerve and glial cells differ well both in chromatin density and in the organization of the cytoplasm. In these terms, the organization of the ganglionic neuropil becomes much more complicated. An increase in the number of their own processes in nerve cells and a weak development of glial membranes leads to the formation in the nerve ganglion, the walls of the rectum, cells and nerve fibers are located compactly, there is practically no free intercellular space. Poorly differentiated cells also lie in compact groups. Nerve and glial cells differ well both in chromatin density and in the organization of the cytoplasm. In these terms, the organization of the ganglionic neuropil becomes much more complicated. An increase in the number of their own processes in nerve cells and a weak development of glial membranes leads to the formation in these terms, the organization of the ganglionic neuropil becomes much more complicated. An increase in the number of their own processes in nerve cells and a weak development of glial membranes leads to the formation in these terms, the organization of the ganglionic neuropil becomes much more complicated. An increase in the number of their own processes in nerve cells and a weak development of glial membranes leads to the formation numerous simple contacts between the former. There are a large number of synaptic contacts, and a significant part of them do not differ in structure from mature contacts. In some cases, one terminal is observed to form several contacts with two or more cells, or by the process of one cell of several contacts with two terminals. Apparently, already at this time, the formation of communication relationships in the neuropil of the ganglion was taking place. Neurons and nerve fibers in the nerve nodes are closely adjacent to smooth muscle cells, and due to the frequent absence of glial layers, they come into contact with muscle cells. Nerve fibers running through the muscle membrane can also form simple contacts with smooth muscle cells.

On preparations in the serous membrane, small single nodules are revealed along the nerve fibers. In the nerve ganglia of the colon, dividing neuroblasts are rare. By 8 months at the age of the fetus, the number of neurocytes is maintained due to the differentiation of neuroblasts into neurocytes, and not by mitotic division. Before the birth of the fetus, the nervous tissue of the colon wall forms a complex intramural nervous system, represented by four large plexuses - intermuscular, submucosal, and actually

mucous. Each of them has a superficial and deep arrangement of ganglia.

By this time, the glial environment of neurons and nerve processes becomes sufficiently developed. A well-developed neuropil of the intestinal ganglia is characterized by the presence of compactly located numerous nerve fibers and profiles filled with various vesicles (Figure 3).

In fetuses at the mid-stage of development of the PNS ganglion, the walls of the colon are located at different levels. Larger ganglia are localized on the annular layer of the muscle membrane, smaller ones - near the muscle plate of the mucous membrane.

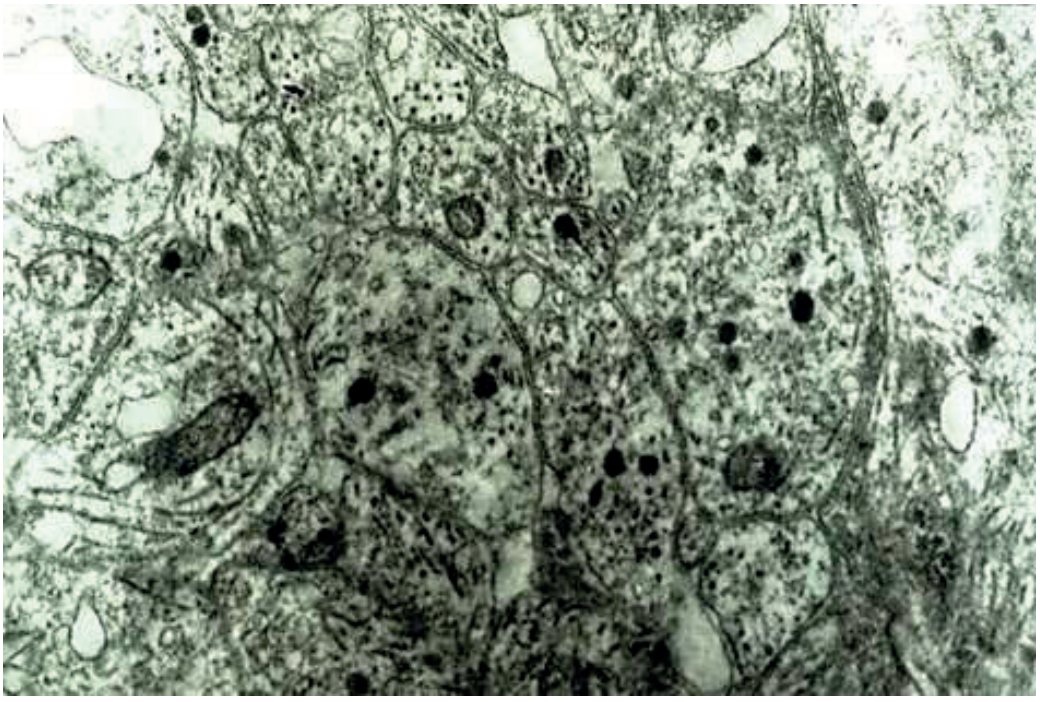

Figure 3. Neuropil of the MHC ganglion of the colon wall of a 9-month-old calf. Zoom 32000

Throughout the fetal and newborn stages of development, the ganglia retain this localization. The ganglia include neuroblasts, single differentiating neurocytes, and neuroglial cells.

Starting from 6-7 months the age of the fetus, in the intestinal wall neuroblasts are found localized in the lamina propria of the mucous membrane of the crypts and the base of the villi.

At the late fetal stage of development, the number of neuroblasts, differentiating neurocytes and neurocytes of types 1 and 2 according to Dogel increases. Some of the nerve fibers are surrounded by the myelin sheath. Under the crypts, in the proper lamina of the mucous membrane, small nerve nodules are found, consisting on transverse sections of 1-2 neurocytes, 3-4 neuroblasts and 9-12 glial cells. These nodules are found in greater numbers in the rectum and cecum than in the colon.

At the late fetal stage of development, the nerve fibers and ganglia of the intermuscular nerve plexus form a large-looped network, and its largest loops are located in the colon, in comparison with the rectum and cecum. Mature neurocytes of types 1 and 2 according to Dogel, in comparison with neuroblasts, differentiating neurocytes and neuroglial cells, have a larger cell area and a high level of development. In the middle and late stages of fetal development, first in the ganglia of the intermuscular nerve plexus, and later in the ganglia of the submucosal nerve plexus, synaptic connections are formed. Polyvalent receptors are found in the mucous and muscular membranes. As a rule, in the ganglia, Dogel type 1 neurocytes

are located separately from type 2 neurocytes. Nerve cells located together, intertwined with dendrites, form dendric tangles. Before birth, as before, glial cells and neuroblasts predominate in the ganglia of the intermuscular and submucosal nerve plexuses. In the serous membrane, along the nerve fibers, there are single nerve nodules.

CONCLUSIONS

The morphological changes observed during the development of intestinal ganglia have the following sequence: 1 - aggregation of neural cells of the anlage of ganglia, coinciding with the growth of preganglionic fibers; 2 - the beginning of differentiation of neural crest cells; 3 - the appearance of cells with glial and neuronal phenotypes (the formation of processes in neural cells); 4 - progressive complication of the shape of neuroblasts, due to the growth and branching of processes; 5 - the appearance of synaptic contacts, ensuring the expansion of interneuronal connections; 6 - some isolation of neurons in the ganglion due to the formation of glial membranes and the connective tissue capsule of the node; 7 - the development of ganglia due to quantitative changes in nerve cells (cell growth, complication of the neuropil, the formation of numerous and diverse synaptic contacts).

The specialization of the intermuscular nerve plexus in black-and-white cattle is formed from 3-4 months, the age of the fetus, and the organ specialization of the submucosal nerve plexus is carried out for 5-6 months.

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NUTRITION

Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

EFFECTS OF USING FERMENTED DUCKWEED ON VOLATILE FATTY ACID AND COLON pH IN RAMBON DUCK

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Abstract

The research was conducted from October to December 2019. Testing samples at the Laboratory of Ruminant Animal Nutrition and Animal Food Chemistry, Animal Husbandry Faculty, Padjadjaran University. This study aims to determine the effect of using fermented duckweed in duck ratio on VFA (Volatile fatty acid) and pH in large intestine. The method used experimentally with a completely randomized design (CRD) with six treatments and four replications. The six treatments were follows: P0 = rations without fermented Duckweed, P1 = rations + 10% Duckweed, P2 = rations + fermented Duckweed 20%, P3 = rations + fermented Duckweed 30%, P4 = rations + fermented Duckweed 40%, P5 = rations + fermented Duckweed 50%. The research data were processed using variance and followed by Duncan's test. The result showed that using 20% of duckweed fermented had a significant effect ($P<0.05$) on the pH large intestine but had no effect ($P>0.05$) on the VFA content.

Key words*: duckweed fermented, pH of the large intestine, Rambon duck, volatile fatty acid.*

INTRODUCTION

Rambon duck is a dual-type duck that can produce eggs and meat. Rambon ducks are ducks originating from the Cirebon area of West Java, the result of a cross between Tegal ducks and Magelang ducks. Ducks are generally bred to produce eggs, but the demand for duck meat continues to increase in Indonesia, so many duck farmers switch their production to produce meat. Various efforts are made by farmers to increase the productivity of meat, one of which is the feed. Morphology of the digestive system of ducks will change in case of a sudden change of feed. Then it is necessary to look for feed materials that have a high nutrient value, among others by utilizing aquatic plants that are processed with fermentation technology, namely fermented duckweed.

Fermented duckweed is a protein source feed material, which will improve the performance of Rambon ducks, the result of the analysis is a crude protein content of 33.84% coarse fiber 8.16% (Setiyatwan et al., 2018). The use of fermented duckweed produces destitute of coarse fiber can be seen in the content of VFA (Volatile Fatty Acid) in the cecum.

The higher the content of VFA means that the coarse fibers in fermented duckweed can be digested. The content of VFA in the duck secretary will affect digestion in the colon because the cellulolytic microbes in the cecum will descend into the colon. These microbes will affect the pH value of the gastrointestinal tract. The use of fermented feed-containing microbes will affect the pH value of the gastrointestinal tract. Fermented duckweed will increase the number of lactic acid bacteria in the colon. The decrease in the pH value in the colon causes the number of lactic acid bacteria to be more and more numerous. The optimum pH value in the colon will increase the number of lactic acid bacteria that will suppress the number of pathogenic microbes so that livestock become healthier, therefore it is necessary to research "Effects of Using Fermented Duckweed on Volatile Fatty Acid and Colon pH in Rambon Duck".

MATERIALS AND METHODS

The study used a day-old duck (DOD) of 120 heads without the straight run. Ducks are placed randomly into 24 cage units and each cage is filled with five (5) heads. Each duck is wing-tagged on the right wing for easy observation and data collection. Ducks are kept from the age of 1 day to the age of 42 days. The cage used is a litter system cage. The cage of 24 flocks is 1 meter x 1 meter x 1 meter per flock for five (5) ducks with the Completely randomized design. The study was conducted experimentally with six (6) treatments and four (4) replications, namely:

P0: Basal ration
P1: Basal ration + duckweed fermentation 10%
P2: Basal ration + duckweed fermentation 20%
P3: Basal ration + duckweed fermentation 30%
P4: Basal ration + duckweed fermentation 40%
P5: Basal ration + duckweed fermentation 50%

The data obtained from the results of the study were analyzed by ANOVA method and further tests of Duncan's.

Table 1. Nutrients Content and Metabolic Energy of Feed Ingredients for the Ration

No.	Feed Ingredient	CP	Cfat	CF	Ca	P	Lys	Meth	Zn	ME
					(%)				ppm	kcal/kg
1	Soybean Meal	41.13	6.36	6.13	0.32	0.67	2.90	0.65	40	2440
2	Kip-Cp144	37.00	2.00	8.16	1.2	1.2	1.36	0.78	72.3	3250
3	Coconut Meal	20.5	8.20	1.31	0.73	0.47	1.26	0.45	0	2540
4	Bran	7.13	11.3	11,8	0.12	1.5	0.34	0.13	30	2400
5	Corn	8.5	7.42	2.86	0.02	0.3	0.22	0.2	18	3300
6	Coconut oil	0	100	0	0	0	0	0	0	8600
7	Premix	0	0	0	23.3	18	0	0	0	0
8	Bone meal	0	0	0	36	0	0	0	0	0

Note: Results of Laboratory Analysis of Ruminant Animal Nutrition and Animal Feed Chemistry, Faculty of Animal Husbandry, Universitas Padjadjaran.

Table 2. Nutrien Duckweed, Duckweed Fermentation and KKK
(Mix Coconut Meal; 35.0%, Soybean Meal; 63.4% and Kip-Cp 144; 1.6%)

Nutrient Content	Duckweed	Duckweed Fermentation	KKK
Crude Protein (%)	30.20	33.84	33.84
Crude Fat (%)	2.39	4.73	6.93
Crude Fiber (%)	19.9%	8.16	4.48
Ca (%)	0.7	0.93	0.48
P (%)	0.4	0.36	0.61
Zn (mg/kg)	76.73	84.60	26.52
Methionine (%)	0.53	0.55	0.58
Cystine (%)	0.14	0.57	0.57
Lysine (%)	0.24	1.11	2.50
Metabolisable Energy (kcal/kg)	2495	2597.4	2487.96

Note: Results from Laboratory Analysis of Ruminant Animal Nutrition and Animal Feed Chemistry, Faculty of Animal Husbandry, Universitas Padjadjaran.

Table 3. Nutrients content and Metabolisable Energy in Treatment

Nutrient	Ransum Treatment					
	P_0	P_1	P_2	P_3	P_4	P_5
Crude Protein (%)	20.74	20.74	20.74	20.74	20.74	20.74
Crude fat (%)	9.44	9.22	8.99	8.78	8.56	8.34
Crude Fiber (%)	4.70	5.07	5.44	5.81	6.17	6.54
Ca (%)	0.56	0.60	0.65	0.69	0.74	0.78
P (%)	0.69	0.66	0.64	0.61	0.59	0.56
Zn (mg/kg)	23.22	29.03	34.84	40.64	46.45	52.26
Lysine (%)	1.37	1.23	1.09	0.95	0.81	0.67
Methionine (%)	0.38	0.37	0.37	0.37	0.36	0.36
Met + Cystine (%)	1.75	1.60	1.46	1.32	1.17	1.03
ME (kcal/kg)	2854.48	2865.42	2876.36	2887.31	2898.25	2909.2

Note: The calculation result of Tables 1 and 2

RESULTS AND DISCUSSIONS

Effect of Treatment on Volatile Fatty Acid (VFA)

Based on the results of the study the influence of the use of fermented duckweed in Rambon duck rations on the content of VFA (volatile fatty acids) can be seen in Table 4.

VFA (Volatile Fatty Acid) is the result of the digestive process of coarse fiber. The process of digestion of coarse fibers in ducks occurs in the cecum. The use of high coarse fiber in rations will affect the digestive process in duck rations. The results of the fingerprint analysis showed t the various levels of use fermented duckweed in Rambon duck rations did not significant (P>0.05) on the content of VFA in the cecum. Although the increase in P1 treatment (142.25 mM) due to the increase in the content of fermented duckweed in rations, but P2 and other treatment did not have an increase in VFA content. This is in line with Mangisah et al. (2005) statement that the use of 15% coarse fiber in duck rations has not increased the absorption of VFA in the cecum.

Based on the analysis gave an idea that the use of fermented duckweed in each treatment at the same range even though the coarse fiber contained in the feed 8.16%. Fermented feed does not affect the content of VFA even though the content of coarse fiber is still quite high. This is in line with Supranoto's research (2000), which tested coarse fibrous rations of 5.10 and 15% and it turns out that the VFA produced in the cecum is also not significantly affected by the treatment. The use of fermented duckweed has not increased the content of VFA in the cecum. The fermented feed has not been able to increase the absorption of VFA. Although fermented duckweed has a high content of crude protein that is 33.84% and has coarse fiber (SK 8.16%). Such coarse fibers for poultry are not very high. This is in line with Sutrisna's research (2010) that the provision of coarse fibers ranging from 5-20% in ducks to fermentative digestion, the total VFA content does not differ markedly.

The results of this study provide information that ducks as one type of monogastric livestock turn out to have the ability to digest coarse fiber into VFA as the final product as in ruminant livestock, although the results are not as much in ruminants. This VFA can be absorbed and utilized as an energy source. VFA fermentation results in cecum can be absorbed and transported to the liver through the port vein. Additional energy generated from the degradation of fibers in the cecum will be used by ducks for basic living and production, one of which is growth. This is by the statement of Nugroho (2000) that the fermentation results can be used as an energy source and help the lack of metabolic energy rations due to increased levels of crude fiber rations.

The increase in crude fiber content in the ration causes the flow rate of the ration in the digestive tract to become fast (Bidura et al., 2008), as a result the digestive tract becomes empty, so that the ducks will consume more rations. In addition, the increase in crude fiber in the ration will reduce the efficiency of metabolic energy use caused by the transfer of some of the net energy fraction for muscular energy activity required for additional activity of the gizard and to push food waste along the digestive tract of chickens (Jhori et al., 1979).

Table 4. Average Content of VFA (Volatile fatty acids) in cecum on various Treatments

Repeat	Treatment					
	P0	P1	P2	P3	P4	P5
	mM					
1	132	141	110	116	139	96
2	125	128	122	112	142	136
3	130	149	114	128	100	99
4	130	151	123	148	151	126
Average	129.25	142.25	117.25	126	133	114.25

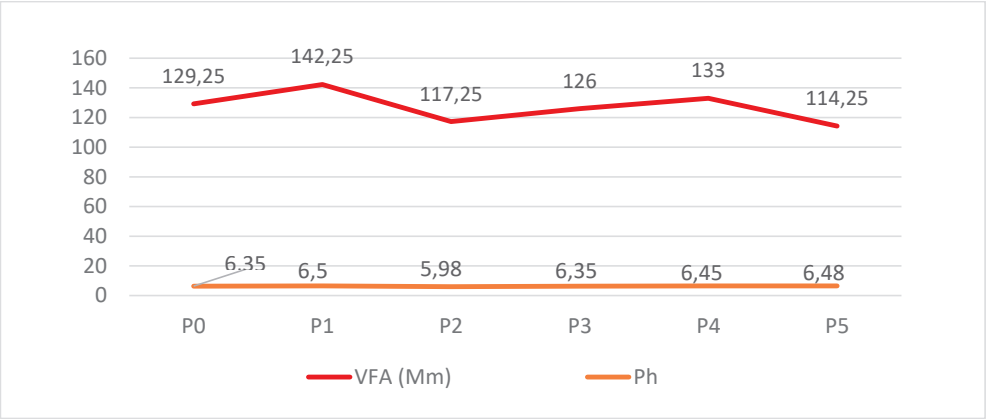

Figure 1. Average VFA and pH Value

Effect of Treatment on colon pH

The colon is the last of the digestive tract before exiting through the cloaca. In the colon, there is the absorption of water and food substances that have not been absorbed by the small intestine. According to McNaught & MacFie (2002), a good colon can be seen from the value of acidity (pH), because if the pH value in the gastrointestinal tract increases or becomes more acidic it will increase lactic acid bacteria. This study to find out the effect of fermented duckweed on the pH of the colon in ducks. The average pH value of the colon of the Rambon duck can be seen in Table 5.

Table 5. Average pH Value of Rambon Duck Colon on various Treatments

Repeat	Treatment					
	P0	P1	P2	P3	P5	P6
1	6.3	6.6	5.9	6.1	6.6	6.5
2	6.5	6.4	5.9	6.3	6.4	6.6
3	6.1	6.6	6	6.6	6.3	6.4
4	6.5	6.4	6.1	6.4	6.5	6.4
Average	6.35	6.5	5.98	6.35	6.45	6.48

Based on the results of the study obtained the pH value of the colon contained ranging from 5.975 - 6.5 with a large average compiled from the lowest treatment P2 (5.975), treatment P0 (6.35), treatment P3 (6.35), treatment P5 (6.45), treatment P6 (6.475), and treatment P1 (6.5). The use of fermented duckweed in the ration of Rambon ducks has a real influence ($P<0.05$) on the pH value of the Colon. It gives an idea that the use of fermented duckweed has an influence on the digestive system of ducks, especially in the colon. Duncan's Double Distance Test was conducted to determine the effect between treatments, the results are listed in Table 6.

Table 6. Duncan Multiple Distance Test Results Effect treatment on pH Value of Large Intestine Duck Rambon

Treatment	average pH value	Significan (0.05)
P_2	5.98	b
P_0	6.35	a
P_3	6.35	a
P_4	6.45	a
P_5	6.48	a
P_1	6.50	a

Description: The different letters in the column significantly indicate the influence of real different treatments ($P<0.05$).

Table 6 showed that the pH of the colon of ducks on the influence of real P2 treatment ($P<0.05$) was lower compared to pH in the treatment of P0, P3, P4, P5, and P1. Based on the results of the Duncan multiple test analysis that P2 has the most influence on other treatments. This shows that 20% of the use of fermented duckweed in Rambon duck rations can decrease the pH value of the colon. Changes in the pH value in the duck colon are influenced by fermented feed and coarse fiber content contained in fermented duckweed, which leads to a decrease in pH value. This is following the statement of Sun (2005) that fermented feed will affect the pH value in the colon. And in line with Sutrisna research (2010), the administration of coarse fiber between 5-20% in duck rations affects the pH value in the colon. The degree of acidity (pH) plays a role in the digestive tract of ducks, such as in the process of re-absorption of food and water substances. The two parameters are interconnected. If the pH value is not in a balanced condition, it will affect the absorption process of food substances, such as B vitamins and water. The pH changes that occurred in the study were due to the presence of microbes in the ration. Fermented duckweed is a feed ingredient that has been done biologically processing by fermenting with *Trichoderma harzianum* and *Saccharomyces cerevisiae* so that there are various types of microbes. This is in line with the statement of Havenaar et al. (1992) probiotics are defined as the living culture of one or more microbes given to farm animals to maintain digestive microflora. The results of research by Hendi et al. (2018) stated that combination with *Trichoderma harzianum* and *Saccharomyces cerevisiae* fermentation can increase the nutritional value of duckweed in terms of increasing crude protein and zinc content and decreasing crude fiber content. Fermented feed that has microbial content in it can affect the pH atmosphere of the colon to increase lactic acid bacteria. Hardiningsih (2006) states that lactic acid bacteria grow optimally at pH 5.8-6.7. According to Widodo (2015), a decrease in pH then bacteria that grow dominated by lactic acid bacteria, bacteria can suppress the presence of pathogenic bacteria. The digestive process in the colon becomes optimal and livestock becomes healthy. The use of fermented duckweed in Rambon duck rations is good for the digestive system of ducks because it does not interfere with microbial activity in the colon.

CONCLUSIONS

Based on the results of the study, it was concluded that the use of fermented duckweed in the ration of Rambon ducks had not been able to increase the VFA content and could reduce the pH value of the large intestine. The use of 20% fermented duckweed has decreased the pH value of the large intestine, the lower the pH value of the large intestine, the fermented duckweed is good for use in the Rambon duck ration.

ACKNOWLEDGEMENTS

The author would like to thank to student researchers and all parties involved in the implementation of this research and funding.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

STUDIES REGARDING THE IMPORTANT FEATURES OF AN ATHLETE'S DIET IN DIFFERENT SPORTS BRANCHES

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Abstract

This work presents the particularities of the diet in some sporting branches depending on the nature of the effort, the organs requested, the climatic conditions etc. Taking into account these criteria, the amount of nutritious substances found in food are established, in order to satisfy the average energetical consumption of a sportsman weighing around 70 kg who practices a certain sport. To help the body recover after exercise sportive must facilitate detoxification and the body's water intake must be maintained at constant limits. The research method presented is based on bibliographic study and experimental methods in order to determine the nutritional values needed for a consistent diet. The sports performances obtained worldwide have reached values that years ago seemed inconceivable. For their achievement, the athletes are subjected to a complex training process, in which the effort often requires the body to exceed its maximum physiological limits.

Key words*: food, nutrients, sports branches.*

INTRODUCTION

In sports, a template of eating is not recommended since it does not reach its target and it is sometimes damaging. To meet the requirements and in order to stay close to these sports activity for as long as possible, it must combine the training process with the observance of the sports life regime, in which the correct nutrition has a primordial role. Hence, an individualisation of the eating pattern is required which needs to be made according to a group of sportsmen or sometimes depending on only one sportsman taking into accound the age, weight, branch of sport. Respecting the life and food regime, both in preparatory stages and especially in the competitive ones, it represents a basic factor that conditions the preparation of the organism at an optimal level and consequently obtaining superior performances. The well-defined substances from a chemical point of view and indispensable to humans are: proteins, lipids, carbohydrates, mineral salts, vitamins and water. From the point of view of the role they play in the body, they are divided into two groups: energetic or caloric and protective or maintenance. The basic substances found in food (proteins, lipids, carbohydrates) have different roles in the body and aid only certain types of effort (Barbuica, 2015). To help the body recover after exercise sportive must facilitate detoxification. Body's water balance must be maintained at constant limits. After the competition when in blood circulating, metabolite results from the effort required a greater amount of water to eliminate. Vegetables and fruits are part of the group that includes all foods of vegetal origin containing lot of water. Vegetables and fruits are a source of vitamin C. The role of vitamin C in the body is very important. It is involved in cellular respiration stimulating redox processes. Enhances the antitoxic action of the liver and increases the overall resistance of the organism. For this reason, sport activity vitamin C should not only be used sporadically or before the start but systematically throughout the training and competitions. During sport activities the need for Vitamin C reaches an average of 150-200

mg per 24 hours, while during the competitional stage may reach 300-400 mg per 24 hours (even up to 500 mg per 24 hours on authors opinion).

MATERIALS AND METHODS

The study regarding the particularities of the sportsman's diet in different sports branches was made using the bibliographic study approach and the experimental method which requires groups of 5 sportsmen from the following branches: athletics, volleyball, basketball, handball, football, rugby and tennis. (Barbuica, 2015)

RESULTS AND DISCUSSIONS

Vegetables and fruits are the most important source of carotene (provitamin A). Highest in carotene content belongs to: leafy greens, carrots, beets, tomatoes, radishes, cherries, cherries, peaches. Vegetables and fruits, in addition to their high vitamin content, also contain minerals. As food predominating alkaline miliequivalents providers they are indispensable for ensuring the acid-base balance of the ration for athletes. Fruit and vegetables are also a source of carbohydrates which, along with vitamins, increase glycogen reserves in liver and improve its functional status.

Vitamins are part of the substances which have an enzymatic role, ferments which aid different chemical reactions, especially the oxidation-reduction ones, acting as catalysts. (Rosoiu, 2003) The minerals are part of all the nutritious substances with a plastic (calcium, phosphorus, potassium and iron salts) and catalytic role (copper, iodine, cobalt, iron salts). They are necessary to the organism in order to maintain its good health and improve the quality of life.

Different types of food used in a sportman's diet as well as the amount of vitamins and minerals can be found in Table 1 (Craciun, 1996).

Athletics is a sport which contains multiple tryouts. Because of this, the food intake needs to be set based on the types of tryouts which have similar features (Banu, 2005).

a) Short distance running, jumping, throwing

Short-term effort with a maximum intensity, emphasising on the reaction speed. The caloric value of the ration reaches 4400-4600 calories in 24 hours and it will be composed of the following nutritious substances:

- Proteins: 170-180 g (60% from animal origin)
- Lipids: 110-120 g (70% from animal origin)
- Carbohydrates: 650-700 g
- Calcium salts (1.8 g), phosphorus salts (3.5 g), potassium salts (3 g)
- Vitamins: B_1 (5 mg) and C (200 mg)

The types of food preferred are: milk, cheese, meat, oatmeal, vegetables (carrots, onion, tomatoes).

b) Middle-distance running

The physical effort is consistent and over a long period of time, with various types of rhythm, emphasising on the resistance based on speed. The caloric value of the ration reaches 4500-5000 calories in 24 hours and it will be composed of the following nutritious substances:

- Proteins: 130-140 g (60% from animal origin)
- Lipids: 130-140 g (70% from animal origin)
- Carbohydrates: 700-750 g
- Vitamins: B_1 (5 mg) and C (30 mg)
- Mineral salts: sodium chloride (10-15 g), calcium (1.5 g), phosphorus (3 g), potassium (3 mg)

The types of food preferred are: milk, cheese, meat, oatmeal, vegetables (carrots, onion, tomatoes).

It is known that although the living matter of the organism seems unchanged, in reality, due to the permanent transformations that underlie life, it is continuously renewed on the basis of substances brought through food.

The metabolism of sports effort shows more important changes depending on the nature of the effort made in different sports, intensity, duration etc.

Table 1. The amount of vitamins and minerals found in different foods for 100 g of product used in a sportsman's diet

Food	Carotene (UI)	Vit. B1 (UI)	Vit. A (UI)	Vit. D (UI)	Vit. K (UI)	Na (UI)	Ca (UI)	Fe (UI)	P (UI)
Cow milk	35	45	150	3-4	160	50	125	0,05	90
Cow Cheese	20	30	50	-	120	30	250	0,5	180
Cottage cheese	0	50	1200	20-40	150	2	500	0,6	400
Rice	30	40	-	-	200	30	15	0,5	150
Pasta	60	120	-	-	140	100	22	1,6	110
Beans	100	700	-	-	1500	60	110	6	400
Peas	150	600	-	-	1000	35	80	5	300

c) Long-distance running

The effort has a medium intensity, but it over a very long period of time. The caloric value of the ration reaches 5500-6000 calories in 24 hours and it will be composed of the following nutritious substances:

- Proteins: 150-160 g (60% from animal origin)
- Lipids: 130-140 g (70% from animal origin)
- Carbohydrates: 750-800 g
- Vitamins: B_1 (5-10 mg) and C (300-400 mg)
- Mineral salts: sodium chloride (20-25 g), calcium (1.5 g), potassium (3.5 mg).

Besides the normal food ration, some food intake is required on the track as well. The products recommended are: sweetened fruit juice (such as lemon, orange, grape), oatmeal concentrates with sugar, salts and vitamins: salted liquid glucose with vitamins, cocoa, sweet and weakened coffee. Tablets containing glucose and vitamin C can also be given to the athletes (Rosoiu, 2003).

Metabolism is the transformation that takes place in living cells based on nutrients when the energy needed for these processes and the development of biological phenomena take place. Metabolism comprises two phases: anabolism and catabolism. Anabolism is the phenomenon of assimilation of nutrients from food and their incorporation in the forms of the human body. Catabolism is the phase of dissimilation and degradation of assimilated substances. The qualitative and quantitative balance of metabolism represents nutrition. It consists of all the phenomena that occur in the body after digestion and absorption of food in the intestine. In order to be useful to the body, food is subjected to transformations, some outside the body and others inside it. Through the digestion process that takes place in the digestive tract, food is broken down under the action of various digestive ferments, first in the substances that are formed (proteins, lipids, carbohydrates, salts, etc.). They are further broken down into simple elements that are absorbed in the intestinal mucosa. After absorption the nutrients pass into circulation and reach the cells where they are metabolized. Since sports effort is achieved mainly with the help of muscles, muscle metabolism must be perfectly adapted to the body's effort, which can be achieved through methodical training, with numerous repetitions of movements in order to form stereotypes.

When it comes to water, after exercise it is not enough just managing salty water but we will add potassium for the diuretic effect. Detoxification can be achieved through a ration with a sufficient intake of water, sodium chloride, potassium chloride, alkaline salts and vitamins, especially B_1 and B_6 a moderate percentage of lipids and carbohydrates, but low in protein. It should be administered 24 hours after the competition. Hypoglycemia resulting from an intense and prolonged effort is accompanied by a decrease in plasma potassium. It is therefore recommended that after an exhausting effort to administrate to the athletes are both carbohydrates and potassium. Carbohydrates needed in the sportive body should be provided at a rate of 65-70% polysaccharides (starch), which gradually digest and does not cause hyperglycemia and only in proportion of 30-35% of mono and disaccharide (glucose, fructose, lactose, sucrose, etc.). Vegetal foods also contain an important polysaccharide called cellulose. This accelerates the intestinal transit in large amounts shorten the time of action of enzymes

on food and absorption during trophies. The amount of cellulose used needs to be higher in the preparatory stages and recovery stages. In competitive stage cellulose intake must be smaller in order to not disturb the digestion. After the competition, the athlete loses a small amount of its reserves of fat. For that ratio to remain balanced and still respect the relationships between foods he can eat: butter, pasta or rice and oil in salads. For recovering of the potassium amount it is recommended the consumption of dried fruit at dinner. Other minerals (magnesium, calcium, iron, etc.) suffer certain changes, but losses may not be compensated immediately.

Carbohydrates reserve should be recovered avoiding massive ingestion of sugars. There are enough carbohydrates ingested at dinner table in form of pasta, rice, potatoes, fruit or fruit juice.

Meat in general and liver in particular have a strong erythropoietic action because these foods contain essential amino acids. Due to the abundance of lysine, meat stimulates the growth process in general, especially muscle growth. Given their nutritional value and mainly their class I protein content, much needed during effort in sports, it is recommended that athletes consume a certain amount of meat or fish per day, especially during speed and strength exercises.

Sports games (volleyball, basketball, handball, football, rugby)

When it comes to sports games, the effort is of a high intensity and it requires speed, strength, resistance and skills. The caloric value of the ration reaches 4500-5000 calories in 24 hours and it will be composed of the following nutritious substances:

- Proteins: 170-180 g (60% from animal origin)
- Lipids: 120-130 g (70% from animal origin)
- Carbohydrates: 650-700 g
- Vitamins: B_1 (3-5 mg) and C (300-400 mg)
- Mineral salts: calcium (2-2.5 g), phosphorus (4-5 g).

A large consumption of fruits and vegetables is required, which besides carbohydrates, contain mineral salts as well as vitamins. The amounts of pasta and bread will be reduced and a consumption of proteins is required, especially coming from an animal origin. These can be found in milk, cheese, meat, fish eggs etc.

Meat, fish and their derivatives along with milk and cheese are a good source of protein with high biological value (Class I proteins). Thus light meat, especially beef, contains 17 to 22% protides, while weak fish contains 15-20% protides. Their association with cereal products raise cereal product's nutritional value. Meat, fish and their derivatives contain significant amounts of minerals. Meat, especially the viscera (liver, kidney) is the richest source of iron. Meat and fish are rich in phosphorus, potassium and sodium but low in calcium. Meat is the most important source of vitamins PP, B_2 and B_6, while fish is a source of vitamins A and D.

Vegetables and fruits are a source of vitamin C. The role of vitamin C in the body is very important. It is involved in cellular respiration stimulating redox processes. Enhances the antitoxic action of the liver and increases the overall resistance of the organism. For this reason, sport activity vitamin C should not only be used sporadically or before the start but systematically throughout the training and competitions.

Vegetables and fruits are the most important source of carotene (provitamin A). Highest in carotene content belongs to: leafy greens, carrots, beets, tomatoes, radishes, cherries, cherries, peaches. Vegetables and fruits, in addition to their high vitamin content, also contain minerals. As food predominating alkaline miliequivalents providers they are indispensable for ensuring the acid-base balance of the ration for athletes. Fruit and vegetables are also a source of carbohydrates which, along with vitamins, increase glycogen reserves in liver and improve its functional status.

The amount of food with composes the ration is determined by the composition of the products as well as the caloric effect produced by 100 g of food. Hence, carbohydrates, lipids and mineral salts are essential. In Table 2 (Alexandrescu, 1994) different products which are part of the sportsman’s ration are presented.

Table 2. Types of food which are part of the sportman's food ration
(proteins, lipids, carbohydrates and their respective calories)

Food	Amount/week	Proteins	Lipids	Carbohydrates	Calories
Cow milk	7 days x 300 g	70	70	96	1350
Cow Cheese	3 days x 100 g	42	4	12	265
Cottage cheese	3 days x 50 g	35	38	-	483
Rice	4 days x 50 g	8	2	135	635
Pasta	4 days x 50 g	26	2	252	776
Peas	4 days x 50 g	10	31	324	1275

Dishes of meat and fish make up sources of vitamins of equal importance as foods originating from. Meat and fish are also a source of energy according to their fat content. The living organism needs food in order to cover energy costs. This energy is expressed by high calories. Depending on the energy requirements of the body we can talk about basal metabolism (basic) and effort metabolism (professional and sport). Diet dominated by meat has the advantage that it allows muscle to increase their volume and strength. Animal proteins stimulate the nervous activity and facilitate the transmission of nerve excitations which consequently help to increase effort capacity especially in the speed contests. In high intensity effort sports (running, throwing, sports games) and in those in which force prevails by imposing a large muscle development (weightlifting, wrestling) 2.3-2.5 g protein per kg of body weight per 24 hours are required. Of these 60% must be of animal origin and 40% of vegetable origin.

It was established that in different sports branches the ideal calorie intake is approx. 5500 calories for 24 hours. In Table 3, the necessity of energetical substances is presented for a 5000 calorie intake over 24 hours as well as their variation in Figure 1.

Given their nutritional value and especially their Class I protein content (necessary during the effort), it is recommended that athletes consume at least 250-300 grams of meat or fish per day especially on speed and strength efforts.

Meat is recommended to be administered on meals before special effort. In the evening meat consumption should be reduced because it can adversely affect the sleep. Meat derivates and canned fish are more nutritious, have a high caloric value, but are harder to be digested. Consuming of large amounts of meat derivates and canned fish determine the change in internal pH to acidic, which is unfavorable for sport activities, especially after the finish of exercises.

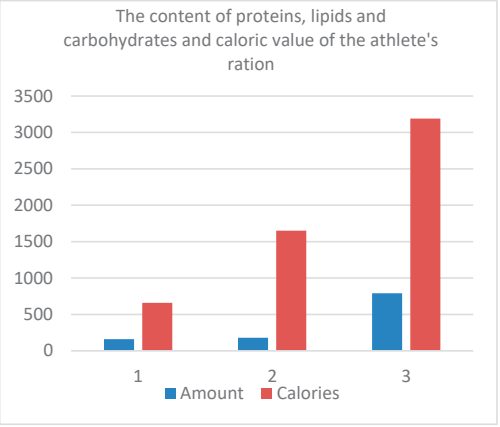

Figure 1. The content of proteins, lipids and carbohydrates and their caloric value present in a sportsman's food ration

Table 3. The required energetical substances in grams for a 5500 calorie intake over 24 hours

12% Proteins	Calories	Grams (g)	Animals origin: 60% = 96 g
	660	160	Plant origin: 40% = 64 g
30% Lipids	1650	180	Animals origin:7 0% = 126 g
			Origine vegetala: 30% = 54 g
60% Carbohydrates	3190	790	Polysaccharides: 65% = 514 g
			Mono and disaccharides 35% = 276 g

Considering vitamin C, this dose must not be exceeded as it may cause various undesirable side effects including sleep, excitement, muscle

cramps. In case of hypovitaminosis C muscle fatigue may occure. This happens more often in winter and spring due to lack fresh vegetables in the diet. Vegetables and fruits should provide 15% of the caloric value of the ration (Rosoiu & Serban, 2003). If this percentage is not reached it is desirable to provide a supplement of Vitamin C athletes as juices. In some studies a correlation between vitamins is required as an excess of one vitamin may influence the effect of another. For example, provitamin A in excess leads to hypovitaminosis C.

CONCLUSIONS

All the vital processes as well as the other human activities are carried out on the basis of an energetic consumption and by incorporating the nutrients. The elements that covers these needs are found in the environment and it is called external food or simply food.

1. In sports practice, it is required for the food intake to be made considering different groups of sportsmen and the following criteria: age, weight and sports branch.
2. Proteins, lipids and carbohydrates have different roles in the organism, supporting different features and types of physical effort.
3. The food intake needs to be organised, taking into account the features of metabolic processes in different tryouts and they are determined by the features of the exchange of substances and the intensity of the physical effort. In the rationed food intake of sportsmen the most reliable correlation is the one between proteins and lipids.
4. A large consumption of fruits and vegetables is required, which besides carbohydrates, contain mineral salts as well as vitamins.
5. Detoxification can be achieved through a ration with a sufficient intake of water, sodium chloride, potassium chloride, alkaline salts and vitamins, especially B_1 and B_6 a moderate percentage of lipids and carbohydrates, but low in protein.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

THE INFLUENCE OF THE FOOD RATION ON THE PROCESS OF MULTIPLICATION AND DEVELOPMENT OF THE INTESTINAL *ENTEROCOCCUS* COMPONENT

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Abstract

The action of food rations on the process of multiplication and development of intestinal enterococci was tested in order to highlight their influence on the health of the intestinal tract. In the study were used four food rations with various caloric structure tested on laboratory animals - white rats, Wistar line. It was established that all the investigated rations differentially influenced the multiplication and development of enterococci. Based on the obtained results, it can be stated that the quantitative indices of enterococci, to a large extent, depend on the composition of food rations. Therefore, we consider that their numerical value can be regulated and maintained not only by microbial preparations with probiotic action but also by the use of food rations, which reflect the prebiotic influence of intestinal enterococci.

Key words*: genus Enterococcus, food rations, multiplication, streptococci.*

INTRODUCTION

Food and its components largely determine the health status of the human organism, in particular through the action on the intestinal microflora.

The intestinal microflora or microbiota is an indispensable component of the digestive tract, being represented by all microorganisms (bacteria, archaea, unicellular eukaryotes like fungi and protozoa, etc.), which through its activity produce various substances / molecules necessary for the normal activity of the human and animal organism (Rowan-Nash et al., 2019).

In general, the intestinal microflora comprises about 50 genera and several hundred species (from 300 to 1000) (Eckburg et al., 2005; Frank et al., 2007). Of these species, only 30-40 constitute the majority (99%) of intestinal bacteria (Sears, 2005). Over 99% of intestinal bacteria are anaerobic, but in cecum, aerobic bacteria reach high densities (Sherwood et al., 2013).

The bacteria of the intestinal microflora are divided into four phyla: *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria*, most of which are attributed to the genera: *Bacteroides*, *Clostridium*, *Faecalibacterium*, *Eubacterium*, *Ruminococcus*, *Peptococcus*, *Peptostreptococcus* and *Bifidobacterium*, and a little part to *Escherichia* and *Lactobacillus*. Numerically dominant bacterial genera are considered to be of major importance in the functioning of the host organism (Khanna et al., 2014).

The concentration and composition of the microbiota varies along the gastrointestinal tract (gut). Only a few species of bacteria are reported in the stomach and small intestine. The large intestine and colon are the most densely populated "habitat" of microorganisms, which can compete with any ecosystem on earth: about 10^{12} microorganisms per gram of intestinal contents (Guarner & Malagelada, 2003; O'Hara & Shanahan, 2006).

The functions of the microbiota are multiple, and its significance is proven by new studies, which demonstrate the close connection between intestinal bacteria and many diseases or conditions we face. The role of the intestinal microbiota in regulating homeostasis, in metabolism, absorption of vital nutrients and synthesis of vitamins, in the formation of immunity, and of the mechanical barrier, which protects the body from harmful agents, has been proven. In general, the intestinal

microflora has multiple physiological roles with resonance on the whole host organism (Hord, 2008).

Observational results over the past two decades suggest that the intestinal microbiota may contribute to the metabolic health of the human host and, and when it is aberrant - to the pathogenesis of various common metabolic disorders, including obesity, type 2 diabetes, non-alcoholic liver disease, cardio and metabolic diseases and malnutrition (Fan & Pedersen, 2021).

The role of the microbiota in the maturation of the immune system was also elucidated, being described the mechanisms of induction and ensuring the functioning of the immune system of the host organism by the intestinal microorganisms (Belkaid & Hand, 2014; Cianci, 2018).

The intestinal microbiota has an essential role in the solubilization of undigested and unabsorbed food residues and their elimination from the body, as well as in the protection against various pathogens by neutralizing toxic compounds (Sekirov et al., 2010; Griffiths, 2015). Intestinal bacteria are a crucial component of the enterohepatic circulation which in turn can influence the metabolization of many drugs, including antibiotics (Gorbach, 1996).

It has recently been shown that by regulating the level and composition of autoantibodies related to appetite-regulating hormones, the microbiota controls aspects of appetite-related behavior and the pathophysiology of eating disorders (Lam et al., 2017).

Consequently, dysbiosis, i.e., qualitative and functional impairment of the intestinal flora, is a serious avenue for understanding the cause of certain disorders, particularly those with underlying autoimmune or inflammatory mechanisms. This has become a central theme in biological and medical research.

On the other hand, the significance of intestinal bacteriocenosis consists in the production of substances (compounds) which in turn have a positive or negative effect not only on the digestive system but also on the whole organism (Gibson and Roberfroid, 1994).

The contribution of the microbiota in the production of various substances, essential amino acids, vitamins, especially B-complex vitamins, necessary for the proper functioning of the host organism, is known. Its role in the production of substances such as dopamine, serotonin or other neurotransmitters, the intestinal microbiota, has also been elucidated, proving the possibility of the microflora to act at a distance (Mangiola, 2016; Swanson, 2015).

The role of protection or biological barrier has been established, by producing short-chain fatty acids (SCFA) and stimulating epithelial regeneration (Belkaid and Hand, 2014; Cianci, 2018). Extracellular metabolites of short-chain fatty acids (SCFA) excreted by the intestinal microbiota have been reported to play an important role in the regulation of intestinal homeostasis. In addition to providing energy, SCFA also causes immune stimulation in animal and human cells (Nakkarach et al., 2021).

There are data on the influence of microorganisms, especially intestinal microorganisms, on the metabolism of the host organism. The microbiota has enzymes that are not encoded in the human genome, but which are needed to perform physiological tasks or to supplement the action of digestive enzymes to break down substances such as polysaccharides, polyphenols. According to this, they can regulate the body's energy balance and cellular metabolism (Baghbani et al., 2020).

The primary role in the digestion of ingested nutrients belongs to the small intestine, as the first region in which ingested food components are subjected to the action of intestinal bacteria, and is the region that is predominantly involved in the digestion and absorption of primary nutrients (Booijink et al., 2007; Leser & Molbak, 2009).

Thus, the microbiota of the small intestine has a major importance for the host (Zoetendal et al., 2012) and an important influence on the physiology and health of the host organism (Cotter, 2011; Duerkop et al., 2009).

Streptococcus and *Veillonella* spp. are the predominant components among the bacterial populations of the small intestine (Bik et al., 2006). *Streptococcus* species are involved in the fermentation of sugars, producing lactic acid as the predominant final fermentation product. In turn, *Veillonella* are famous for

their ability to use lactic acid as a source of carbon and energy (Ng & Hamilton, 1971).

During the past century, the classification of the genus *Streptococcus* has been refined, with the most significant change occurring in 1984 when some species of bacteria of the genus *Streptococcus* were separated into two genera: *Enterococcus* and *Lactococcus* and most members of the Group D streptococci, including *Streptococcus faecalis* and *Streptococcus faecium*, were included in the new genus *Enterococcus* (Schleifer & Kipper, 1984).

The genus *Enterococcus* includes lactic acid bacteria - Gram-positive cocci with high potential for colonization of various habitats, including the digestive tract in animals and is characterized by increased resistance to extreme pH values, ionizing radiation, osmotic and oxidative stress, at high concentrations of heavy metals and antibiotics, as well as at temperatures up to 45°C (Vu & Carvallo, 2011). Only *E. faecalis*, *E. faecium*, *E. avium*, and *E. durans* can colonize the human intestine, but two most common species are *E. faecalis* (90-95%) and *E. faecium* (5-10%) (Ramsey M. et al., 2014).

Enterococci are a model for studying the influence of food rations on the intestinal microflora and how the food consumed can contribute to the health of the digestive tract and the host organism through their action on the microbiota (Tannock & Cook G., 2002). They are also used in studies on how the body copes to coexist with a variety of beneficial and harmful strains of the same species, probably managing to select the ones that are more advantageous. These bacteria, as highly evolved commensals, have been extensively used in the food industry and as probiotics to prevent or ameliorate disease (Ramsey et al., 2014; Sánchez et al., 2019). *Enterococcus* is therefore a good model of how certain diets can protect the host, promoting, or not, the growth of strains with different levels of safety once they have reached the intestine (Penders et al., 2006; Timoşco et al., 2015).

Based on the above mentioned, it is proposed that it would be rational to use intestinal enterococci in the development of new preparations for probiotic use.

Thus, the aim of the paper was to study the action of different food rations (developed for the first time in the Institute of Physiology and Sanocreatology, Republic of Moldova) on the process of multiplication and development of intestinal enterococci, which are of interest to the food and pharmaceutical industry.

MATERIALS AND METHODS

In order to reveal the impact of the new developed food rations on the multiplication and development of intestinal streptococci, two experiments were performed. In both experiments, the new food rations elaborated for first time at the Institute of Physiology and Sanocreatology were tested. The structure of the developed rations is reflected in Table 1.

Table 1. Caloric structure of newly developed rations, **%**

Basic indices	Variants of food rations					
	1	*2*	*3*	*4*	*5*	*6*
Proteins	8	9	10	11	12	14
Lipids	35	33	31	29	27	25
Carbohydrates	57	58	59	60	61	61

The first experiment was performed *in vitro* and aimed to highlight the rations that have a more pronounced effect on enterococci development and multiplication. This experiment included seven lots, being tested 6 new food rations developed, as follows: Lot I - control, in which the inoculation of enterococci was performed separately on nutrient medium Enterococco Agar (Aesculin Azide Agar Balls) (https: //assets.thermofisher.com/TFS-Assets/LSG/manuals/IFU1194.pdf).; lots II-VII - experimental lots, in which the inoculation of enterococci was performed together with the decoction of six food rations (Table 1).

The second experiment was performed *in vivo* using laboratory animals (white rats, Wistar line). In this experiment, the action of food rations (preventively selected in the *in vitro* experiment) on the process of multiplication and development of intestinal *Enterococcus* bacteria was tested. The structure of the experiment is as follows:

- Lot I - control (administration of food ration no. 1);
- Lots II-VII - experimental (administration of food rations no. 4, 5 and 6).

For this purpose, samples of intestinal (rectal) contents were collected from all animals in two stages: at the beginning and end of the experiments. The samples were subjected to

research using classical microbiological methods (Garmasheva & Kovalenko, 2010). Their inoculation was performed on agarized elective nutrient medium, recommended for enterococci (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/IFU1194.pdf).

Over 72 hours after incubation of the inoculated samples on Petri dishes at 37 ± 1ºC, quantitative indices of enterococci were calculated at 1 g of intestinal contents (by multiplying the number of colonies by diluting the sample). The final results are expressed in decimal logarithms (log) (GOST 30518-97, 2000).

RESULTS AND DISCUSSIONS

As above mentioned, food rations or diet have a direct action on the intestinal microflora. Modulation of nutrient concentrations in diets may have a differential influence on the intestinal microbiota, including *Enterococcus* species.

The results obtained in *in vitro* experiments (Table 2) reveal the differentiated action of the new food rations on the quantitative indices of enterococci.

Table 2. Numerical value of bacteria of the genus *Enterococcus* inoculated *in vitro* separately and in common with newly developed food rations

The lot	The way of inoculation *separately, **in common with 6 food rations	The amount of live microbial cells per 1 ml of suspension, logarithms decimal (log/ml)	The difference to control, %
I	*	8.69 ± 0.65	
II	**1	6.38 ± 0.39	-26.58
III	**2	6.63 ± 0.39	-23.70
IV	**3	6.17 ± 0.48	-28.99
V	**4	8.59 ± 0.63	- 1.15
VI	**5	8.77 ± 0.67	+ 0.92
VII	**6	8.50 ± 0.64	- 2.18

The obtained data demonstrate that the inoculation of enterococci separately on elective nutrient medium ensured their multiplication up to the quantitative level of 8.69 log/ml. The decoction of six variants of newly developed food rations contributed to obtaining of different results. Thus, in the lots, where were tested the rations with no. 1, 2, 3, which is characterized by a higher concentration of lipids, the number of microbial cells is lower compared to Lot I. Food ratios no. 4, 5 and 6 (with a higher concentration of proteins and carbohydrates) ensured a quantitative level of microbial cells identical to that of Lot I (control). It follows that the testes food rations acted on the process of multiplication of enterococci differently, ensuring various levels of development of these bacteria (from 6.17 to 8.77 log/ml) (Table 2).

It should be noted that in the performed experiments bacteria of the genus *Enterococcus* showed different sensitivity to the primary composition of food rations.

Thus, an inhibitory action on enterococci manifested food rations no. 1, 2 and 3. It was established that the ration no. 1 (containing 8% proteins, 35% lipids and 57%) and no. 3 (containing 10% proteins, 31% lipids and 59%) had the greatest effect of numerical inhibition of bacteria. The rations no. 4 and 6 also contributed, to a lesser extent, to the numerical reduction of these microorganisms. Food ration no. 5 (containing 12% proteins, 27% lipids and 61% carbohydrates) acted as a stimulant, contributing to the non-essential increase in the number of bacteria of this genus.

Therefore, based on the obtained results, it was found that the tested food rations had a different action on the process of multiplication and development of bacteria of the genus *Enterococcus*. The rations no. 1, 2 and 3 showed an inhibition action on microorganisms, and the ration no. 5 had a stimulating action on multiplication of bacteria. Thus, it can be stated that the quantitative indices of enterococci largely depend on the composition of food rations. Therefore, we consider that their numerical value can be regulated and maintained not only using the microbial preparations with probiotic action but also through the diet with different structure of components (nutrients), which reflect the prebiotic influence of intestinal enterococci.

In order to confirm the *in vitro* results, *in vivo* experiments were performed on white laboratory rats, Wistar line. For *in vivo* testing of the action of food rations on intestinal enterococci, rats were grouped into four experimental groups (lots). In the first lot, it was administered the food ration, containing 8% proteins, 35% lipids and 57% carbohydrates (ration 1); in lot II - the ration with the structure of 11% proteins, 29% lipids

and 60% carbohydrates (ration 4); in group III - the ration with the structure of 12% proteins, 27% lipids and 61% carbohydrates (ration 5) and group IV - the ration containing 14% proteins, 25% lipids and 61% carbohydrates (ration 6).

In general, the rations that showed action to stimulate the numerical growth of intestinal enterococci (in *in vitro* experiments) were selected.

The ration with the structure of 8% protein, 35% lipids and 57% carbohydrates served as a control (control lot).

The structure of the tested rations and the grouping of laboratory animals according to the experimental lots are indicated in Table 3.

Table 3. Quantitative characteristic of newly developed and *in vivo* tested food rations, %

Basic components	The quantity, %, according to the variants of the tested food rations/ number of experimental lots of animals			
	1/I	4/II	5/III	6/IV
Proteins	8	11	12	14
Lipids	35	29	27	25
Carbohydrates	57	60	61	61

The body mass of the experimental animals and the quantitative indices of *Enterococcus* were determined as a result of the tests.

The experimental data were noted at the beginning and end of the experiments (after 60 days of administration of food rations) and are reported in Tables 4 and 5.

Table 4. Body mass of rats used to experiment with various food rations

The lot	Weight of rats in g/1 animal, according to the time of determination		Weight gain g/1 animal	Difference to the beginning, %
	at the beginning	at the finally		
I	242.4 ± 17.96	325.2 ± 26.70	82.8	34.15
II	242.8 ± 13.49	356.2 ± 22.58	113.4	46.70
III	242.0 ± 15.36	368.8 ± 21.60	126.8	52.39
IV	242.8 ± 12.22	352.6 ± 29.64	109.8	45.22

The analysis of the data obtained on the body mass of the tested animals revealed the positive impact of the tested food rations, with varied nutritional value. The lowest increase in body weight (by 34.15%) during the administration of the tested rations was established in group I, which served as a control. The other variants of food rations tested (rations no. 4, 5 and 6) contributed to an increase in body weight, during their administration, respectively by 46.70%, 52.39% and 45.22%. Based on the data on body mass (weight gain) of laboratory animals, it was found that the most optimal tested ration proved to be food ration no. 5, which was administered to the animals in group III.

Next, the action of the tested rations on bacteria of the genus *Enterococcus* was determined, as a component part of the intestinal microbiota.

Based on obtained data (Table 5) it was established that the ration tested in group I (control) contributed to the increase by 69.27% of the final numerical indices of facultative microorganisms of the genus *Enterococcus*, which indicates the abundant development of these bacteria.

Table 5. The modification of the numerical indices of *Enterococcus* bacteria in the intestinal contents of rats, fed with different nutritional value food rations

The lot	The amount of microbial cells per 1 g of intestinal contents, decimal logarithms (log)		The difference, %	
	at the beginning	at the finally	Comparative to the beginning	Comparative to the lot I
I	5.11 ± 0.36	8.65 ± 0.42	+69.27	
II	5.50 ± 0.39	6.58 ± 0.48	+19.63	-23.93
III	5.67 ± 0.41	6.63 ± 0.39	+16.93	-23.35
IV	5.23 ± 0.22	6.17 ± 0.41	+17.97	-28.67

In the animals from experimental groups II, III and IV, during the administration of the tested rations, a non-essential increase of the number of microbial cells was observed. Thus, the numerical value of the researched bacteria increased respectively by 19.36%, 16.39% and 17.97%. However, compared to the animals from lot I, the numerical indices of enterococci decreased respectively with 23.93%, 23.35 % and 28.67%.

Consequently, the results obtained in *in vivo* conditions, when the food rations were testing on laboratory animals, do not confirm the data obtained in *in vitro* conditions, when food rations were testing on nutrient medium. The differences in the data obtained in *in vivo* and *in vitro* conditions, indicate that in the intestine of animals, bacteria of one or another kind of genera of obligative or facultative microorganisms do not act separately, but in

association. The antagonistic influence of the representatives of the intestinal microflora is most frequently manifested. In particular, enterococci are inhibited by *Lactobacillus* representatives. On the other hand, it is known that among intestinal enterococci, the species *E. faecalis* predominates quantitatively compared to *E. faecium,* and increasing of their number is not beneficial to the host organism.

The data regarding the action of diets on the gut microbiota are quite heterogeneous. This is largely determined by many factors such as the duration of diet administration, the structure of nutrients in food rations, the model organism studied and the types of analyzed microorganisms.

What is certain, is that the nutrient structure of the food rations has a direct action on the microbial composition (Li et al., 2009; Holscher et al., 2018; Johnson et al., 2019).

However, it is considered to have a positive impact those diets that contribute to maintaining the „ecological homeostasis" of intestinal microorganisms (Leeming et al., 2019).

Importance to maintain a constant level of enterococci derives from their property to produce a wide variety of bacteriocins often called enterocins. They are also active against Gram-positive foodborne pathogens, such as *L. monocytogenes* (Izquierdo et al., 2009). *E. faecium* and *E. faecalis* are the main producers of enterocins. Bacteriocin-producing probiotics could compete with intestinal pathogens for colonization or modulate the microbiota homeostasis (Salvucci et al., 2012; Cotter et al., 2013). Enterococci, due to the property of producing bacteriocins, can be used as a probiotic with beneficial effects on the health of the host organism.

Thus, the fact that the tested food rations, *in vivo* conditions, do not conduct to a large numerical increase of enterococci, indicates to their positive impact.

Therefore, we consider that their numerical value can be regulated and maintained not only through utilization of microbial preparations with probiotic action but also by using food rations, which reflect the prebiotic influence of intestinal enterococci (on the example of tested ration no. 5).

Thus, experimentally it was found that the dietary factor (tested food rations) during the entire investigation contributed to the optimization of the content of enterococci in the intestine of model animals.

CONCLUSIONS

It was found that the tested food rations show different action on the process of multiplication and development of intestinal enterococci.

The quantitative indices of enterococci depend to a large extent on the composition of food rations.

Among the tested rations, the ration with no. 5 had the best result, in terms of numerical modification of enterococci and based on data regarding the body mass of the tested animals.

Numerical value of enterococci can be regulated and maintained not only by microbial preparations with probiotic action but also by the use of food rations, which reflect the prebiotic influence of intestinal enterococci

ACKNOWLEDGEMENTS

This research work was carried out with the support of Institute of Physiology and Sanocreatology and also was financed from Project 15.817.04.01A.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

PRODUCTION AND EVALUATION OF EXTRACELLULAR ENZYMES FROM *BACILLUS LICHENIFORMIS* IN DIFFERENT RAW MATERIALS USED IN ANIMAL FEED

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Abstract

The production of different enzymes, including amylase and protease by Bacillus licheniformis ATCC 21424 were tested on different raw materials and compound feed, which were used as a substrate in process fermentation: soybean meal, peas, sorghum flour, corn and compound feed (FC). The bacterial growth and enzyme production were done in a fermentative medium (Erlenmeyer 100 ml flash in a shaking incubator) and enzyme activity was registered after 24, 48 and 72 h, pH 7 ± 2). The inoculum strain presents 10.19 log CFU/ml at 37°C, 24 h, 150 rpm. The screening showed a capacity of amylase and protease strain production. The highest amylase activity was obtained when the strain was cultured in corn fermentation medium (19.43 U/ml), followed by soybean meal (18.31 U/ml), sorghum (17.52 U/ml), peas (19.43 U/ml), and FC (6.63 U/ml) at 72 h. Great protease activity was noticed in FC (97.75 U/ml), soybean meal (94.67 U/ml), sorghum (89.36 U/ml), corn (78.6 U/ml), peas respectively (75.91 U/ml). The observation of this study suggested that Bacillus licheniformis ATCC 21424 could be capable of producing protease and amylase enzymes, particularly in fermented medium contained soybean meal or corn, and can be administrated in animal nutrition as source of feed additive.

Key words*: Bacillus* spp.*, extracellular enzymes, enzymatic activity, raw materials.*

INTRODUCTION

The European Commission (EC) decided to stop the utilization of feed antibiotics as growth promoters in the animal live cycle (2006) due to their capacity to transfer resistance genes between bacteria decreasing the beneficial intestinal flora (Hmani et al., 2017).

Exogenous feed enzymes occur as an essential solution in animal nutrition by acting against antinutritional factors (e.g., β-glucans, pentosans, phytate) with positive effects on dietary components digestion (Slominsky, 2011; Ciurescu et al., 2020), digesta viscosity, nutrients utilization, pathogens inhibitor by equilibrating the host intestinal microflora (Chen et al., 2013; Dumitru et al., 2020a), improving, in the end, the productive animal performance (Ravindran, 2013).

Bacteria from *Bacillus* group can involve beneficial biotechnological products, with industrial applications, including a source of probiotics in animal nutrition (Dumitru et al., 2020a, 2020b; Ciurescu et al., 2020). Probiotics were well-defined as live cultures of bacteria or yeasts with positive actions in the host (Chen et al., 2013).

Due to their ability to synthesize enzymes, *Bacillus* spp. are known for the production of amylases and proteases, together representing more than 70% of the total important enzymes (Mukhtar & Haq, 2012). *Bacillus* spp. are used in industrial animal production, enhancing the absorption of the nutrients and reducing the *Salmonella* spp. (Ghorban Hosseini et al., 2018) and *Escherichia coli* biotype β-hemolysis infections during the gastrointestinal tract (GIT) (Dumitru et al., 2020a).

Blanco et al. (2016) affirmed that optimization of the fermentation process is based on the selection of a suitable culture medium including substrate composition, incubation time, temperature, pH medium (most enzymes have the optimum pH between 4 to 6), carbon and nitrogen source type, and agitation conditions. Regarding the high rate of sporulation and growth, bacteria as *Bacillus amyloliquefaciens, Bacillus subtilis, Bacillus licheniformis,* and *Bacillus stearothermophilus* (Far et al., 2020) have been shown the

capability of producing quantitatively α-amylase for industrial applications, due to their property to resist at high temperatures. Generally, enzyme activities increase up to 40°C, but quickly, a zone of decline is recorded due to the denatured and loss of their structure recorded with an inactivation for the enzyme of interest.

The current study is based to test the potential of *Bacillus licheniformis* ATCC 21424 for evaluating the capacity to produce and secrete hydrolytic amylase and protease enzymes in the different fermented medium as a new criterion for use as probiotics in animal feed.

MATERIALS AND METHODS

The *in vitro* assessment was performed at the Biotechnology Laboratory of IBNA Balotesti and all procedures complied with the experimental protocol.

Bacterial culture

Bacillus licheniformis (BL) was acquired from the American Tissue Culture Collection (ATCC 21424) as a freeze-dried form. After revitalized in nutrient Merck broth medium (g/l: tryptone 10; meat extract 5; sodium chloride 5; pH medium 7.2 ± 2 before autoclaving) and incubated at 37°C for 24 h in aerobic conditions, the stock culture was maintained at 4°C on Merck nutrient agar slants (g/l: tryptone 5; meat extract 3; bacteriological agar 5; distilled water). For long preservation, the strain was stored at -80°C with 20% sterile glycerol.

Medium preparation, inoculum and fermentation process conditions

Inoculum was prepared as follows: some colonies of a 24 h old slant *BL* culture were transferred with a sterile inoculation loop into a tube containing 9 ml of sterile broth medium under aseptic conditions.

The amylase was produced in **submerged fermentation medium (SFM)** consists of (g/l): glucose 6%, $CaCl_2$ x $2H_2O$ 0.04%, $MgCl_2$ 0.02% with the addition of 2% raw materials (soybean meal, peas, sorghum flour, corn and combined feed).

The inoculated culture was incubated at 37°C, 24 ± 2 h, 150 rpm (in a shaker-incubator) follow by inoculation (1:10, v/v) into 250 ml Erlenmeyer flasks containing 100 mL of autoclaved (121°C, 15 min) SFM. After an incubation time of 24, 48 and 72 h at 37°C under continuous shaking (150 rpm), each fermentation medium with the addition of corresponding raw materials was centrifugated at 8000 rpm, 20 min, 4°C in a cooling centrifuge. The supernatants obtained were used for quantitative estimation for enzymatic activity.

Each fermentation process was done in triplicate and optical density (OD) was measured following the enzymatic method.

In vitro assessment of amylase activity

Qualitative method

BL strain was tested for amylase activity by starch hydrolysis test using the Iodine method. A nutrient agar medium with the addition of 1% (w/v) starch following by sterilization at 121°C for 15 min was prepared. The inoculated plates were incubated at 37°C for 24 h. After incubation, the starch hydrolysis zone was detected by inundating the plates with 3-5 ml of iodine solution (Lugol Grams straining or 0.4% KI + I_2, w/v). The blue color development showed the presence of starch, while the areas around the hydrolytic bacteria appeared clear (Dumitru et al., 2018).

Quantitative method

Enzymatic activity. For evaluation of amylase activity (AA), DNS (3,5-dinitrosalicylic acid) spectrophotometric assay was used. As an alkaline reagent, DNS attaches to the reducing sugars, following be measurements of the color changes by UV absorbance at 546 nm with a BioSpectrometer Basic Eppendorf. The cell-free supernatant recovered represents the crude enzyme extract. The reaction mixture containing: crude enzyme extract (0.5 ml) with 0.2 M phosphate buffer (0.5 ml) for pH 7.0 and 1% (w/v) soluble starch prepared in 0.2 M phosphate buffer (1 ml) previously maintained at 30°C/10 min. The reaction was stopped immediately adding 2 ml of DNS following by 5 min of boiling. After cooling, the sample was diluted with distilled water (up to 12 ml/tube) and absorbance was measured at 546 nm.

Standard curve of maltose

A maltose standard curve was done to determine the quantity of reducing sugars in the

reaction mixture. One unit of amylase activity (enzymatic units, U/ml) was defined as the amount of enzyme/ 1 ml of culture supernatant that released 1 µmole of reducing sugars as maltose/min under the assay conditions.

In vitro assessment of protease activity

Qualitative method

Bacillus licheniformis ATCC 21424 was screened for protease-producing bacteria. After inoculated on the nutrient agar with addition of skim milk (1% w/v), the plates were incubated at 37°C, for 48 h, in anaerobic conditions. After incubations, the strain protease capacity was evidential by clear zones around colonies. Zone of hydrolysis was done by flooding agar plate with 25% TCA (trichloroacetic acid) solution and incubated for 15 min, at 45°C (Dumitru et al., 2018; Siddalingeshwara et al., 2010).

Quantitative method

Enzymatic activity. The production of protease in culture filtrate was estimated by the Anson method. Method is based on the determination of tyrosine, resulting from the action of proteases on the casein substrate.

The sample test consists of 1 ml of casein (1% enzymatic substrate prepared in 0.2 M phosphate buffer, pH 7) added to 0.5 ml crude enzyme solution. The reaction mixture was well homogenized and incubated for 10 min at 35°C. Then 2 ml of TCA reagent (5%, w/v in distilled water) was added, shaken to mix very well and filtrate with filter paper. In another tube, was taken 1 ml from filtered solution over which was added 2 ml 0.5 N NaOH and 0.5 ml Folin-Ciocalteu (FC, 1:3 v/v in distilled water) reagent, mixed and incubated 10 min at 35°C. To enhance the amylase production of *BL*, the fermented medium was incubated at 37°C, 150 rpm followed by measuring the enzymatic activity at 24, 48 and 72 h. OD of the solutions was determined at 660 nm.

Standard curve of tyrosine

A tyrosine standard curve was effectuated to quantify the amount of protein in the SFM. One unit of protease was defined as the enzymatic activity that releases 1 µmole of tyrosine from 1 ml of SFM in one minute.

Statistical analysis

All results were performed in triplicates and the results were done using analysis of variance (one-way ANOVA) as a completely randomized design, 2011). The results are expressed as mean values and standard error of the mean (SEM). Data were analyzed by STAT VIEW for Windows (SAS, version 6.0), the differences between means were considered statistically significant at $P<0.05$, using Fisher's PLSD test for the untitled compact variable.

RESULTS AND DISCUSSIONS

Bacterial culture

Before testing the enzymatic capacity of *Bacillus licheniformis* ATCC 21424 to synthesize extracellular enzymes as amylase and protease on various raw materials feed (soybean meal, peas, sorghum flour, corn and combined feed), the present strain was subjected to several tests for evaluating their potential as a source of probiotics in animal nutrition. Morphological and biochemical characterrization done by Dumitru et al. (2019) indicated the strain potential with other assays as growth rate, percentage of survivability at low pH, bile salts concentrations, high temperatures, hemolysis activity etc. involving positive results. Furthermore, the active 24 h culture *BL* was tested for their carbohydrate fermentation using API 50 CHB system kit (BioMerieux, France) respecting the manufacturer's instructions. After a visual examination at 24, 48 and 72 h, Dumitru et al. (2019) reported the API 50 CHB, results obtained after the color change from red to yellow and their capacity to synthesize enzymes. As could be seen in the report of Dumitru et al. (2019), *BL* has the capacity to ferment the starch and lactose substrate present in API 50 CHB kit as confirms enzymatic status to produce amylase and protease.

Amylase activity assay

Qualitative method

During the present study, *BL* secrets amylase in agar medium in the presence of 1% starch, property observed by the development of a hydrolysis zone around colonies.

Optimization of culture conditions is an essential aspect for enhances bacteria growth and enzyme production. The enzymatic system of *BL* to secrete amylases can be observed by discoloration of the nutrient agar medium supplemented with soluble starch at the addition of

Iodine solution. It is very important to know what is the strain capacity to secretes enzymes and to do that, the substrate-specificity must be selected carefully.

The introduction of starch into the nutrient medium accelerated the *BL* strain system for amylase secretion that decomposes the highly specific substratum.

Amylase enzyme was observed by a hydrolysis zone around colonies developed on agar medium. Another study conducted by Deb et al. (2013), reported similar results in case of *Bacillus amyloliquefaciens* P-001 as a source of amylase production.

Quantitative method

To determine the amylase activity of our strain, a standard curve of maltose was done (Figure 1). The extracellular amylase activity of *BL* strain was carried out in shake-flask fermented medium using different raw materials used in animal-based diet: soybean meal, peas, sorghum flour, corn and combined feed (FC) in a percentage of 2%. The results can be observed in Table 1.

As can be observed, in the first 24 h of fermentation, *BL* when grown in fermented media with corn flour exhibited a higher capacity to secrete amylase, following by soybean meal, sorghum, peas and FC, registering statistically differences ($P < 0.05$) between all substrate used as a carbon source. This finding is very helpful in animal feed.

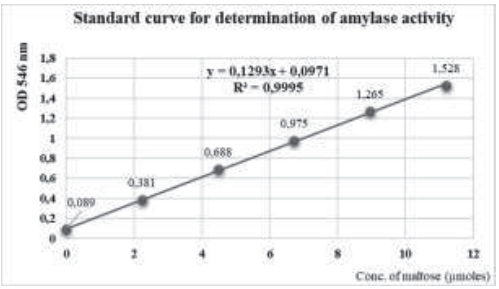

Figure 1. Maltose standard curve for determination of amylase activity

The highest *BL* amylase activity was at 72 h in FC fermented medium, while the strain capacity produces 19.43 U/ml.

Because the presence of starch in raw materials encourages the production of amylase, during the entire period of fermentation, AA was synthesized by *BL* strain to hydrolyze the starch present in the medium and to produce more easily assimilable sugars (dextrins and progressively smaller polymers composed of glucose units and maltose, Abdel-Fattah et al., 2013; Blanco et al., 2016).

Table 1. Extracellular amylase production from *Bacillus licheniformis* ATCC 21424 in shake-flask cultivation

Item	Amylase activity [U/ml]						
	[S]					*SEM**	P-value
T	**Soybean meal**	**Peas**	**Sorghum**	**Corn**	**FC**		
24h	13.11^{aA}	6.96^{bA}	11.34^{aA}	14.75^{dA}	3.54^{cA}	1.10	0.0001
48h	16.77^{aB}	10.05^{bB}	$14.^{43bB}$	5.09^{dB}	17.88^{eB}	1.26	0.0001
72h	18.31^{aC}	11.60^{bC}	17.52^{cC}	6.63^{dC}	19.43^{eC}	1.30	0.0001
Effect							
[S] × T							
SEM**	0.77	0.68	0.89	0.44	0.69		
P-values	0.0001	0.0001	0.0001	0.0001	0.0001		

Where: FC, combined feed; *BL, Bacillus licheniformis* ATCC 21424; [S], raw material used as substrate; T, time (h); SEM, standard error of the means in a row; $^{a-c}$Means within same rows with different superscript letters are significantly different ($P < 0.05$); $^{A-C}$Means in same collumns between rows with different superscript uppercase letters are significantly different ($P < 0.05$).

After 48 h, the amylase production in corn fermented medium was decreased comparatively with soybean meal. For us, an important place consists in AA evolution in FC medium, the formula which will be given to the animal as a feed source. Considering that, at 72 h, *BL* strain had a considerable growth rate in FC medium, soybean meal, sorghum, peas and corn, AA increasing in parallel with time of incubation ($P < 0.05$).

In the present work, *Bacillus licheniformis* was found as an effective enzyme producer through submerged fermentation process.

As source of energy, corn represents the main ingredient in animal diet, and together with sorghum, are known as no viscous cereals (Ravindran, 2013). In our study, corn has 3.353 kcal/kg of metabolizable energy (ME), 87.63% of it is dry matter, 7.11% crude protein and 3.86 cellulose (Habeanu et al., 2017). For example, NRC (2012) presented from the ME of corn, 62.6% of it is starch and 9.7% non-starch polysaccharides (NSP) as indigestible components. The addition of enzymes as amylases aims to degrade the presence of indigestible components and can contribute to better digestion of starch and therefore to intensify the nutritional value of feed and energy in animal nutrition (Hmani et al., 2017).

It is known that sorghum is a non-viscous grain with high protein content (9.91%, Habeanu et al., 2017; Shargie, 2020) and antinutritional factors (i.e., tannins, phytate) that may form stable complexes with proteins and minerals which reduces digestibility and nutritional value (Schons et al., 2012). Addition of exogenous amylase (derived from *Bacillus* spp. as affirmed Mahagna et al., 1995) and protease in sorghum-based broiler diets enhanced the total tract digestibility of amino acids. Of relevance of the present study is that the amylase production in sorghum fermented medium at 72 h of incubation was higher than corn substrate due to the *BL* activity to degrade starch into maltodextrins and simple sugars. Between all raw materials used, corn medium realised the maximum level of glucose in early stage of fermentation (24 h) due to starch-hydrolysing effect of activated amylase.

Soybean meal (SBM) is one of the commonly used protein source for animal feed with a high NSP content, factors which diminish its utilization (Mukherjee et al., 2015). Incorporation of *BL* as a microbial enzymatic source in fermented medium with soybean meal involved a strong interaction. *BL* as can be observed in Tables 1 and 2, has the capability to improve nutritional value of SBM

registering 18.31 U/ml amylase, but with a significant level of the protease of 94. 66 U/mL in 72 h of fermentation. Hong et al (2004) confirmed that the addition of microbial fermentation using bacteria or fungi efficiently improves the nutritional value of SBM by eliminated the anti-nutritive compounds. Besides, Han et al. (2001) and Yang et al. (2007) affirmed that *Bacillus* spp. are preferred to produce fermented soy-based feed.

Peas are usually utilized in nonruminant diets as excellent source of protein. For swine, field peas present average of 23% crude protein and 3.435 kcal/kg digestible energy compared to corn (NRC, 1998). Peas contain anti-nutritional factors (i.e. galactosidase, trypsin inhibitors, resistant starch, pectin, tannins, lectin, phytic acid) and NSP which significantly damage the digestive process especially in young chicks (Goodarzi Boorojeni et al., 2017). The addition of microorganisms in fermentation processes could provide several benefits with enzymatic probiotic effects of animal GIT. The use of microbial strains with enzymatic properties as an additive in animal feed could inactivate anti-nutritional factors, enhance the digestion process and nutrient availability (Bedford, 2000).

In GIT of poultry, the retention time of feed is very short (~2 to 4 h) and this interval can fluctuate in function of the chemical and physical characteristics (i.e. particle size, feed form administration, NSP concentration, etc.).

On the other hand, the optimum pH of most exogenous enzymes is between 4 and 6. Therefore, the GIT pH and the fact that enzymes can be exposed to hydrolysis by endogenous proteolytic enzymes in the GIT, the degradation activity of exogenous enzymes seems mainly limited in the crop, proventriculus and gizzard (Ravindran, 2013).

The inclusion of *BL* in peas fermented medium improves the level of amylase and protease enzymes (Tables 1 and 2) where were registered significant enzymatic activities (72 h).

Several studies confirmed that younger animals, in the first days of life, due to an immature digestive system, do not produce sufficient endogenous digestive enzymes to degrade NSP present in cereals which involves a negative effect and decreases the nutrients digestibility (Adami dos Passos & Kim, 2014; Song et al., 2010). The addition of amylase enzyme catalyses the presence of endohydrolysis linkages NSP in simple units as glucose, form which can be absorbed by the animal body. As an energy source of growth, amylases complement bird endogenous enzyme secretion, increase starch digestibility and

diminish the availability of glucose as a potential substrate for non-beneficial bacteria in the latter part of the animal GIT (Anguita et al., 2006). It was observed that supplementation with *Bacillus licheniformis* ATCC 21424 (Dumitru et al., 2020a) in piglets diet based on 33.48% corn, 25% sorghum, 17% peas, 13% soybean meal improved growth performance and intestinal microflora population. Furthermore, positive modifications of the entire study were observed with significantly results on diarrhoea incidence of piglets. In most cases, studies with addition of *Bacillus* as a source of microbial enzymes may be a suitable alternative and have been found to involve high benefits in the reduction of bacterial gastrointestinal diseases in animal feed (Ibrahim et al., 2012; Lattore et al., 2016).

Protease activity

Qualitative method

On solid medium supplemented with skim milk, the Petri plate revealed a clear zone around colonies sign the capacity of strain to produce protease. Protease enzyme is very important in the process of digestion due to the capacity to hydrolase proteins and anti-nutritional factors (Hmani et al., 2017).

Quantitative method

With regard to protease production, a standard curve of tyrosine was done (Figure 2).

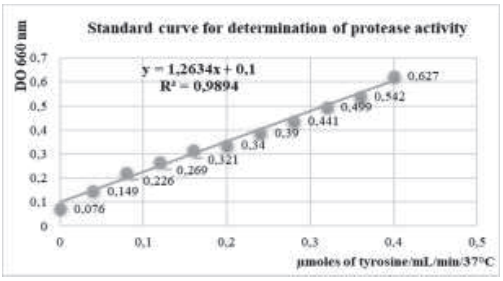

Figure 2. Tyrosine standard curve for determination of protease activity

The strain capacity to secrets proteases enzymes was followed in fermented media with different sources of raw materials used as substrates (Table 2). The protease activity (PA) was determined by quantifying the amounts of tyrosine released from casein hydrolysis by incubating the fermented media time of 24, 48, and 72 h.

Table 2. Extracellular protease production from *Bacillus licheniformis* ATCC 21424 in shake-flask cultivation

Item	Protease activity [U/ml]					*SEM**	P-value
	[S]						
	Soybean meal	Peas	Sorghum	Corn	FC		
24 h	2.57^{aA}	2.19^{bA}	3.46^{cA}	2.69^{adA}	3.02^{eA}	0.11	0.0001
48 h	72.50^{aB}	68.46^{bB}	73.06^{acB}	70.68^{abdB}	94.27^{eB}	2.52	0.0001
72 h	94.66^{aC}	75.91^{bC}	89.36^{cC}	78.60^{dC}	97.75^{eC}	2.13	0.0001
Effect							
[S] × T							
SEM**	13.87	11.71	13.17	15.51	12.04		
P-values	0.0001	0.0001	0.0001	0.0001	0.0001		

Where: FC, combined feed; *BL, Bacillus licheniformis* ATCC 21424; [S], raw material used as substrate; T, time (h); SEM, standard error of the means in a row; $^{a-e}$Means within same rows with different superscript letters are significantly different ($P < 0.05$); $^{A-C}$Means in same collumns between rows with different superscript uppercase letters are significantly different ($P < 0.05$).

Protease production increased gradually. In all fermentation cultures, the evolution of protease synthesis was improved from 24 to 72 h of incubation. Individually, each raw material due to the presence of proteins induced the production of proteases during the fermentation process at the addition of *BL*; the strain acted and degraded them in small components (proteins to peptides and amino acids). *BL* showed the highest protease production at 72 h of incubation in fermented medium with FC, followed by soybean meal, sorghum, corn, and peas used as enzymatic substrates ($P < 0.05$). Production of protease varied among raw materials used with *BL* addition. A littlest decrease in protease enzymatic activity at 72 h was found for peas (< 28.77%) and corn (< 24.36%) compared to FC fermented medium ($P < 0.05$). These results correlate with Blanco et al. (2016) report. Hmani et al. (2017)

affirmed that protease-amylase combination determined significant improvements in the animal growth performance, body weight gain (Garcia et al., 2008), total protein digestibility and reduction in gas emissions (Bundgaard et al., 2014). Enzymes such amylase, protease, cellulose etc. are generally produced by *Bacillus* spp.; an addition to an animal-based diet is known to involve beneficial effects on digesta viscosity, body weight, feed intake, nutrient absorption (Schallmey et al., 2004).

CONCLUSIONS

The results obtained showed that the present raw materials could be used for enzyme (amylases and proteases) production by *Bacillus licheniformis* ATCC 21424. The addition of 10% (v/v) inoculum in different fermented medium gives appreciable results at 72 h to produce and secrete significant quantities of extracellular enzymes as amylase (corn, soybean meal, sorghum, peas and FC) and protease (FC, soybean meal, sorghum, corn and peas). Hence its *BL* can be recommended as an enzymatic source for animal nutrition.

ACKNOWLEDGEMENTS

The present study received financial support UEFISCDI through the project 8PCCDI/2018-PC 2, and part of activity received technical support from Romanian Ministry of Education and Research by Project No. PN 19.09.01.04.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

CHANGES IN THE HAEMATOLOGICAL PROFILE OF ROMANIAN BLACK AND SPOTTED DAIRY CALVES

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Abstract

The aim of this study was to evaluate the haematological profile of Romanian Black and Spotted dairy calves during the first three months of life. Forty dairy calves, clinically healthy, from the Experimental Farm of the Research and Development Institute for Bovine Balotesti were screened for hemoleukogram (CBC) evaluation using the Abacus Junior Vet 5 haematology analyser. Comparisons between age groups (t_1: 3-7 days; t_2: 30 days; t_3: 60 days; t_4: 90 days, n = 10 heads/age group) were performed using One-Way ANOVA with post hoc Tukey test. Significant differences in the erythrogram components for platelets (PLT) and mean platelets volume (MPV) were observed (PLT: t_1 vs t_2: $p = 0.0001$; t_1 vs t_3: $p = 0.0014$; t_1 vs t_4: $p = 0.0000$; MPV: t_1 vs t_3: $p = 0.0148$). Statistical differences for leukocytes (WBC) and monocytes (MO) were also observed (WBC: t_1 vs t_2: $p = 0.0397$; t_1 vs t_3: $p = 0.0214$; MO: t_1 vs t_3: $p = 0.0284$). The main blood parameters studied were significantly different during the first three months of life of the calves, expressing a haematological adaptation pattern of the un-weaned dairy calves.

Key words*: age, cattle, dairy calves, haematological adaptation, hemoleukogram.*

INTRODUCTION

The hemoleukogram (CBC) was found to offer valuable information in the diagnosis, surveillance, and estimating of a prognosis regarding the progression of a disease in an individual (Jones & Alison, 2007; Roland, 2014). The study of haematological profile of newborn dairy calves during the adaptation period to the environmental challenges and immunological immaturity is important (Novo et al., 2015), given that this crucial neonatal period is characterized by high morbidity and mortality (Mee, 2008). The values of different blood variables in calves and other young animals are changing with age (Mohri et al., 2010; Moosavian et al., 2010; Brscic et al., 2015). Additionally, the geographical position, management factors, breed of the animal and laboratory factors can affect the haematological reference intervals (Mohri et al., 2007; George et al., 2010). Changes can be observed in the haematological profile of calves due to the transition from the intrauterine environment to the external environment (Knowles et al., 2000; Benesi et al., 2012a). The reference values of different blood variables are well established for adult cattle, however, for calves, there is a scarcity on data available. This study evaluated the haematological profile of healthy newborn calves from birth up to 3 months of age.

MATERIALS AND METHODS

All experimental procedures were performed in accordance with the *Romanian Law no. 43/2014* and the *Council Directive 2010/63/EU* regarding handling and protection of animals used for scientific purposes.

Forty dairy calves (Romanian Black and Spotted, n=10 heads/age group), from the Experimental Farm of the Research and Development Institute for Bovine Balotesti, were screened for hemoleukogram (CBC) during the first three months of life. The blood samples were taken at the following time: t_1: 3-7 days, t_2: 30 days, t_3: 60 days, and t_4: 90 days. The calves were housed in individual hutches and received a daily ration of 6 liters of milk, divided in two meals per day. Alfalfa hay, concentrate feed, and water were offered *ad libitum*. Blood samples were collected aseptically from the jugular vein (1-2 ml) of each animal, in vacutainer tubes with anticoagulant using disodium ethylene diamine tetra acetic acid (EDTA). Haematological parameters (red blood cells count, haemoglobin concentration, hematocrit percentage, red blood

cells distribution width, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, platelet percentage, mean platelets volume, platelets distribution width, total white blood cells count, lymphocytes percentage, monocytes percentage, neutrophil percentage) were determined using the automated hematology analyzer Abacus Junior Vet 5 (Diatron, Hungary). The analyses were carried out in the Animal Physiology and Biochemistry Laboratory of the institute. Results were expressed as a mean (± standard deviation). Comparisons between age groups were performed using One-Way ANOVA with post hoc Tukey test. Significance was declared when $p < 0.05$ and $p < 0.01$.

RESULTS AND DISCUSSIONS

The mean and standard deviation of the erythrogram components are shown in Table 1. No significant effect between the studied age groups ($p > 0.05$) was found for RBC ($F_{(3; 39)} = 0.88$; $p = 0.4602$), HGB ($F_{(3; 39)} = 1.45$; $p = 0.2440$), HTC ($F_{(3; 39)} = 1.40$; $p = 0.2560$), and RDW ($F_{(3; 39)} = 0.57$; $p = 0.6369$). The obtained value for red blood cell count (RBC) was $9.36 \pm 1.24\ 10^6/\mu l$ in the first 7 days of life (t_1) and decrease to $8.95 \pm 1.0710^6/\mu l$ at the 90 days of life (t_4). The HGB concentration ranged from 10.58 ± 1.72 g/dl at 7 days of life (t_1) to 9.48 ± 0.79 g/dl at 60 days of life (t_3), and 10.12 ± 0.95 g/dl at 90 days of life (t_4). The obtained HCT percentage ranged from $30.80 \pm 5.04\%$ at 7 days of life (t_1) to $27.24 \pm 2.49\%$ at 90 days of life (t_4). After the first week, RDW increased till $26.31 \pm 3.48\%$ at 60 days of life (t_3) and decreased further to $24.67 \pm 3.78\%$ at 90 days of life (t_4). The current results are not in accordance with those reported by Baccili et al. (2018) in Holstein calves. Moreover, no changes in erythrogram components in calves from birth up to 30 days of life were observed by Benesi et al., 2012a. Mohri et al. (2007) reported that the HGB, MCH, and MCHC decrease during the first month of life and then start to increase up to the age of 3 months.

Table 1. Mean values of RBC, HGB, HTC and RDW in healthy calves from birth up to 3 months of age

Period/Haematological indicators	RBC, $10^6/\mu l$	HGB, g/dl	HTC, %	RDW, %
	X ± sd	X ± sd	X ± sd	X ± sd
t_1	9.36 ± 1.24	10.58 ± 1.72	30.80 ± 5.04	24.66 ± 2.93
t_2	8.57 ± 0.95	9.59 ± 1.61	28.13 ± 5.36	24.65 ± 3.55
t_3	8.88 ± 1.11	9.48 ± 0.79	27.24 ± 2.49	26.31 ± 3.48
t_4	8.95 ± 1.07	10.12 ± 0.95	29.74 ± 3.48	24.67 ± 3.78
X	8.94 ± 1.09	9.94 ± 1.36	28.98 ± 4.32	25.07 ± 3.39

RBC = red blood cells count; HGB = haemoglobin concentration; HCT = hematocrit percentage; RDW = red blood cells distribution width; t_1: 3-7 days; t_2: 30 days; t_3: 60 days; t_4: 90 days.

The obtained average values for MCV, MCH, and, MCHC (Table 2) were not different statistically ($p > 0.05$) between the studied age groups (MCV: $F_{(3; 39)} = 1.21$; $p = 0.3195$; MCH: $F_{(3; 39)} = 0.71$; $p = 0.5494$; MCHC: $F_{(3; 39)} = 0.52$; $p = 0.6661$).

Table 2. Mean values of MCV, MCH, and MCHC in healthy calves from birth up to 3 months of age

Period/Haematological indicators	MCV, fl	MCH, pg	MCHC, g/dl
	X±sd	X±sd	X±sd
t_1	32.7 ± 2.79	11.29 ± 0.84	34.39 ± 1.32
t_2	31.9 ± 3.30	10.36 ± 0.90	34.24 ± 1.46
t_3	30.9 ± 2.64	10.77 ± 1.04	34.87 ± 1.54
t_4	33.5 ± 3.78	11.44 ± 1.41	34.16 ± 1.20
X	32.45 ± 3.18	11.16 ± 1.06	34.42 ± 1.36

MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; t_1: 3-7 days; t_2: 30 days; t_3: 60 days; t_4: 90 days.

Variations for MCV and MCH were detected by a gradual decrease of their values at the first week of life up to 30 days of life (MCV:31.9 ± 3.30 fl; MCH:10.36 ± 0.90 pg). This may coincide with the replacement of foetal HGB with adult HGB. The decrease in HTC and MCV in the first month of life can be considered as transient microcytic anemia,

considering the reference ranges for adult cattle (Mohri et al., 2007). The MCHC showed similar mean values between 34.39 ± 1.32 g/dl (t_1) and 34.16 ± 1.20 g/dl (t_2). Novo et al. (2015) reported the following values: 36.4 ± 1.6 fl for MCV, 11.3 ± 0.8 pg for MCH, and 31.0 ± 2.0 g/dl for MCHC in calves at 30 days of life.

The means and standard deviations of the PLT, MPV and PDW are shown in Table 3. A significant differences between age groups for platelets (PLT: $F_{(3;\ 39)} = 11.23$; $p = 0.0000$), and mean platelets volume (MPV: $F_{(3;\ 39)} = 3.52$; $p = 0.024$) were observed (PLT: t_1 vs t_2: $p = 0.0001$; t_1 vs t_3: $p = 0.0014$; t_1 vs t_4: $p = 0.0000$; MPV: t_1 vs t_3: $p = 0.0148$). The obtained results in this study indicate that PLT count increase rapidly in the first week of life, after which the dynamics of PLT count differ. The number of platelets (PLT) in calves was 681.9 ± 204.47 10^3/µl in the first 7 days of life (t_1), decreased rapidly to 353.4 ± 149.31 10^3/µl at 30 days of life (t_2), then slowly increased at 408.5 ± 91.94 10^3/µl (t_3), and decrease slightly to 338.9 ± 137.62 10^3/µl at 90 days of age (t_4). No statistically significant changes were noted in the PDW percentage ($F_{(3;\ 39)} = 1.60$; $p = 0.2040$). In dairy calves mean values recorded for PDW were 33.69 ± 2.26% at t_1, decreased (30.67 ± 3.42%) at t_3, and increased (31.57 ± 4.16%) at t_4. Panousis et al. (2018) published mean values of 603.8 ± 294.6 10^9/l for PLT, and 7.77 ± 1.48 fl for MPV in calves.

Table 3. Mean values of PLT, MPV and PDW in healthy calves from birth up to 3 months of age

Period/Haematological indicators	PLT, 10^3/µl	MPV, fl	PDW, %
	X ± sd	X ± sd	X ± sd
t_1	681.9 ± 204.47	5.86 ± 0.44	33.69 ± 2.26
t_2	353.4 ± 149.31	5.67 ± 0.31	32.59 ± 2.83
t_3	408.5 ± 91.94	5.29 ± 0.36	30.67 ± 3.42
t_4	338.9 ± 137.62	5.59 ± 0.47	31.57 ± 4.16
X	445.67 ± 202.199	5.60 ± 0.44	32.13 ± 3.32

PLT = platelet count; MPV = mean platelets volume; PDW = platelets distribution width; t_1: 3-7 days; t_2: 30 days; t_3: 60 days; t_4: 90 days.

The means and standard deviations of the leukogram components are shown in Table 4. Statistical differences for the total white blood cells count (WBC: $F_{(3;\ 39)} = 3.81$; $p = 0.0180$) and monocytes (MO: $F_{(3;\ 39)} = 3.37$; $p = 0.0287$) were observed (WBC: t_1 vs t_2: $p = 0.0397$; t_1 vs t_3: $P = 0.0214$; MO: t_1 vs t_3: $p = 0.0284$). A gradual decrease of the total number of white blood cells with the increase of age was observed (t_1: 11.75 ± 2.55 10^3/µl; t_2: 9.09 ± 1.94 10^3/µl; t_3: 9.82 ± 2.28 10^3/µl; t_4: 8.85 ± 1.61 10^3/µl). Lymphocytes ($F_{(3;\ 39)} = 1.64$; $p = 0.1955$) showed value of 53.01 ± 16.64 % in the first 7 days of life (t_1), followed by an increase, with maximum values (64.86 ± 5.67%) observed at 60 days of life (t_3), and a decrease (57.25 ± 19.09%) at 90 days of life (t_4). Monocytes grow in the first 7 days of life (t_1: 8.97 ± 10.51%), followed by a gradual decrease (t_2: 2.88 ± 3.03 %; t_3: 1.5 ± 0.68%) then they are no longer influenced by age. Values between 35.15 ± 7.94% and 39.6 ± 16.55% for neutrophils during the first 90 days after birth (t_4) were recorded ($F_{(3;\ 39)} = 2.20$; $p = 0.1043$).

Table 4. Mean values of WBC, LY, MO and NE in healthy calves from birth up to 3 months of age

Period/Haematological indicators	WBC, 10^3/µl	LY, %	MO, %	NE, %
	X ± sd	X ± sd	X ± sd	X ± sd
t_1	11.75 ± 2.55	53.01 ± 16.64	8.97 ± 10.51	35.15 ± 7.94
t_2	9.09 ± 1.94	51.61 ± 13.69	2.88 ± 3.03	45.52 ± 12.20
t_3	9.82 ± 2.28	64.86 ± 5.67	1.5 ± 0.68	33.66 ± 5.36
t_4	8.85 ± 1.61	57.25 ± 19.09	3.15 ± 3.11	39.6 ± 16.55
X	9.87 ± 2.34	56.68 ± 15.03	4.12 ± 6.19	38.48 ± 11.85

WBC = total white blood cells count; LY = lymphocytes percentage; MO = monocytes percentage; NE = neutrophil percentage; t_1: 3-7 days; t_2: 30 days; t_3: 60 days; t_4: 90 days.

Leukogram variations in calves within the first week of life could be caused by the stress following the postpartum adaptation, caused by the elevation in circulating levels of endogenous glucocorticoids in the blood of the calves and their effects on different blood leukocytes (Benesi et al., 2012a). In this case, the leukocyte formula can acquire a neutrophilic profile. In the current study, the leukocyte formula had a lymphocyte profile. The total means values of the hemoleukogram (CBC) components in the dairy calves at 0-3 months of life are shown in Figure 1. The recorded values in this study were not in accordance with values reported by Parvu et al. (2003) in un-weaned dairy calves.

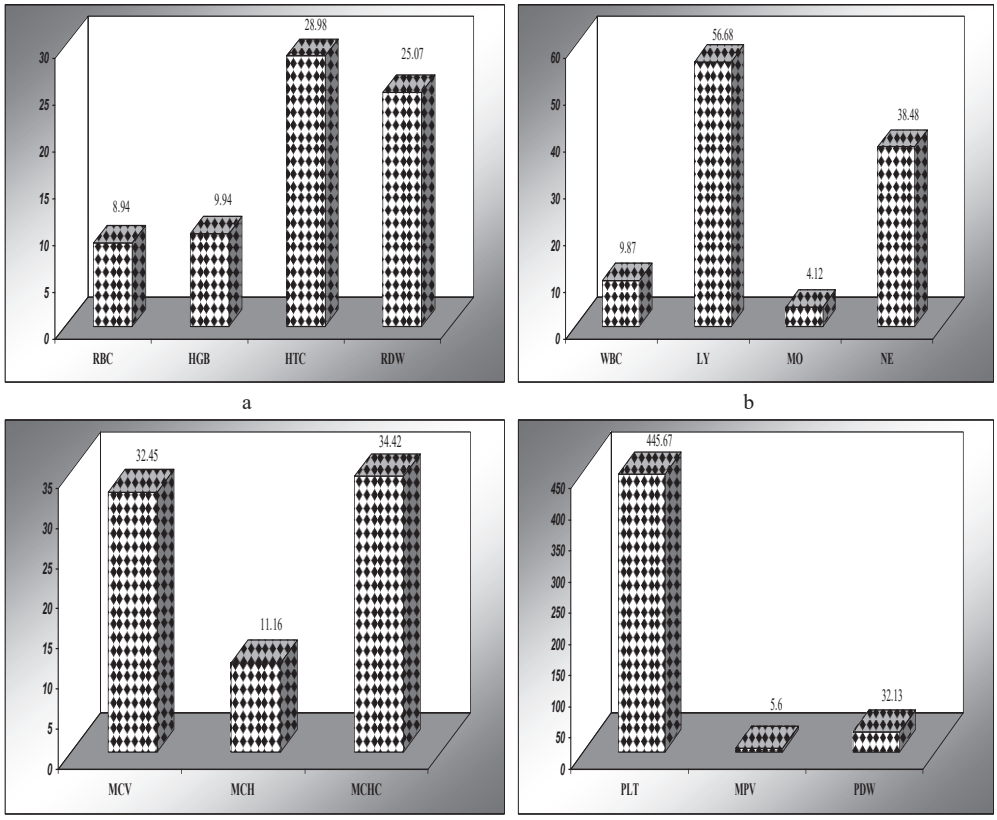

Figure 1 a, b, c, d. Mean values of hemoleukogram (CBC) in healthy dairy calves at 0-3 months of life

Botezatu et al. (2014) reported the following values: 9.1 10^6/mm^3 for RBC, 12.5 g/dl for HGB, 34.2% for HCT, 42 μ^3 for MCV, 12.8 pg for MCH, 35.5 g/dl for MCHC, 454 10^3/mm^3 for PLT, 8.1 μ^3 for MPV, 9.1 10^3/mm^3 for WBC, 65% for LY, 0.65% for MO, and 34% for NE, in Holstein-Friesian calves between 0 to 3 months. In comparison with adult cows' reference values reported by Parvu et al. (2003), calves had lower mean of HGB, HTC, MCV, MCH, higher mean of RBC, WBC, NE, and similar value for LY and MO. Klinkon and Jezek (2012), Jonson and Alison (2007), Brun–Hansen (2006) reported that, in young calves, RBC counts and HTC percentage might be higher, and, MCV and MCHC might be lower than in adults cows. The total white blood cells count (WBC) in blood of calves is higher comparative with adult animals and is more variable as values of other haematological variables. Different types of leucocytes have different life spans so their number can change rapidly and blood serves only as transport medium from the place of origin to the place of inflammation (Roland, 2014; Kraft, 1999a). In young animals, the number of leukocytes,

including neutrophils is higher than in adults. With age, this situation changes, the number of neutrophils decreasing in favour of lymphocytes (Abramowicz et al., 2019).

CONCLUSIONS

The obtained results, showed significant variations in the components of the CBC of calves during the first 3 months of life. These haematological variations could be a consequence of the stress related to environmental adaptation challenges that calves are facing.

Current results, collected with those of other authors, could represent a first step in setting up reference values for the haematological profiles in clinically healthy un-weaned dairy calves.

ACKNOWLEDGEMENTS

This study was supported by Research Internal Thematic Plan of the Research and Development Institute for Bovine Balotesti, Project No. 4466/2018 "Research regarding metabolic profile in cattle and buffaloes".

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

RESEARCH ON THE EFFECT OF A DIETARY SUPPLEMENT ON GROWTH AND ERYTHROGRAM IN PIGEONS

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Abstract

Pigeon breeding is an activity with tradition in Romania, because their meat is a very valuable food product. The aim of the current paper is to investigate the effect of a dietary supplement (containing trace elements, vitamins and amino acids) used by pigeon fanciers for growing pigeon chicks. Another goal is to investigate whether the dietary supplement has effects on the erythrogram of pigeon chicks. The obtained results showed that the dietary supplement, due to its components, determined an intensification of the growth rate of pigeon chicks, so it can be used successfully for this purpose. Regarding the erythrogram, there were observed significant increases in the number of erythrocytes and hemoglobin (which demonstrates an intensification of erythropoiesis) and decreases in MCV and MCH (which demonstrates that the intensification of erythropoiesis is accompanied by the installation of microcytosis and hypochromia).

Key words*: amino acids, erythrogram, pigeon, trace-elements, vitamins.*

INTRODUCTION

Pigeon breeding is an activity with tradition in Romania and their meat is a very valuable food product (Savu et al., 2002; Petcu, 2015; Costachescu et al., 2019; Paraschiv et al., 2020; Okoh et al., 2020). Nowadays, there is a wide range of dietary supplements on the market that can be administered to pigeons in different situations (growth, convalescence, during training, during the cold season, after the administration of treatments, during the mating season, before competitions, etc.). Their beneficial effects are obvious.

The effects of these supplements on the growth rate of pigeon chicks and on the erythogram are not so well known.

The aim of the current study is to find out how the food supplement Selevit sol. influences the growth rate of pigeon chicks and also if it has effects on the erythrogram.

We consider this study as a novelty for those interested in raising pigeons because the obtained values (the body weight of the pigeon chicks) can be used as reference values for pigeon fanciers who use dietary supplements in their own farms. At the same time, the values of the erythrogram resulting after the use of the dietary supplement and especially the explanations of the obtained results may be useful in the future in carrying out other research on similar topics.

MATERIALS AND METHODS

The biological material was represented by 20 pigeon chicks of the standard carrier breed. They were split in 2 lots (each lot being made up of 10 pigeon chicks), resulting a control group and an experimental group. The dietary supplement used in the current research was Selevit sol. (contains vitamins, amino acids and trace elements).

The working methods used in the research were: determination of pigeon chicks body weight, determination of erythrocyte count (RBC), hemoglobin dosing (Hb), determination of hematocrit (HCT) and determination of derived erythrocyte constants (MCV, MCH, MCHC).

In order to **determine body weight,** an electronic Myria scale was used on the day of hatching and on days 7, 14, 21 and 28 of the experiment. Blood samples were taken on the 28th day to determine the erythrogram.

The erythrocyte count (RBC) was determined by direct counting using a hemocytometer.

The dosing of hemoglobin (Hb) and hematocrit (HCT) was performed using the HemoSmart device.

Determination of average erythrocyte volume (MCV), determination of average erythrocyte hemoglobin (MCH) and determination of average erythrocyte hemoglobin concentration (MCHC) were obtained using the calculation formula recommended by the literature (Cotor et al., 2012; Ghiță, 2010).

The current research was performed on 2 experimental groups, as follows:

- group 1: control group (no food supplement was administered);
- group 2: experimental group (0.1 ml dietary supplement was administered orally, daily, during the experiment).

RESULTS AND DISCUSSIONS

Results and discussions on the evolution of body weight

Regarding the evolution of the body weight of the pigeon chicks, the obtained results are presented in Table 1 and in Figure 1, accompanied by comments and discussions (explanations and comparisons with the data found in the literature). We specify that the obtained results will be presented as average values for each experimental group. Comparisons on the statistical relevance of the differences between the experimental groups were made using the t (Student) test.

Table 1. Average body weight values in pigeon chicks from the 2 experimental groups, expressed in grams

The experiment's day	Group 1	Group 2
1st day	16.4	15.9
7th day	67.5	72.4*
14th day	171.3	189.2*
21th day	346.6	412.2*
28th day	424.1	574.7*

*p<0.05

Analyzing the data presented in Table 1, it is observed that on the first day of the experiment the average body weight of the pigeon in group 2 is 3.05% lower than the average body weight of the pigeons in the control group, the difference not being statistically significant. This statement confirms that the 2 experimental groups were constituted correctly.

On the 7th day of the experiment, the average body weight of the pigeons in group 2 is higher than the average body weight of the pigeons in the control group by 7.26%, the difference being statistically significant ($p<0.05$). The increase body weight is probably due to trace elements.

Studying the literature (Dojană et al., 2019) we found data that were related to the intensification of the growth rate of youth in all species, so it can be deduced that pigeons were included. An explanation of the obtained results seems to be represented by the time from the introduction of dietary supplement, which was probably insufficient for the vitamins and amino acids (from the dietary supplement) to be effective.

On the 14th day of the experiment, the average body weight of the pigeon chicks in group 2 is higher than the average body weight of the pigeon chicks in the control group by 10.45%. On the 21st day of the experiment the average body weight of the pigeon chicks in group 2 is 18.93% higher than the average body weight of the pigeon chicks in the control group. On the 28th day of the experiment the average body weight of the pigeon chicks in group 2 is higher than the average body weight of the pigeon chicks in the control group by 35.51%.

In all three situations mentioned above, the differences are statistically significant ($p<0.05$). These differences of the average body weight are due to vitamins, amino acids and trace elements (the trace elements are involved in the metabolism of carbohydrates, lipids and proteins, by participating in various anabolic reactions as enzyme activators) (Cotor et al., 2006; Pop et al., 2006).

The dietary supplement can be successfully used when the aim is to increase the body weight of pigeon chicks because, in order to obtain a maximum effect, it is necessary to supplement the diet with vitamins, amino acids and trace elements.

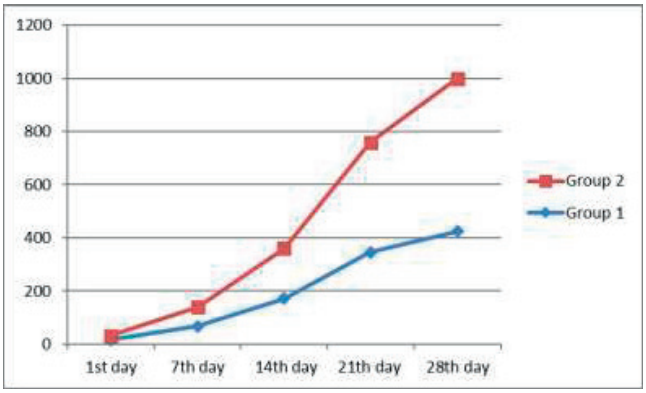

Figure 1. Variation of body weight of pigeon chicks during the experiment

Results and discussions on erythrogram values

The obtained results are presented in Table 2 and in Figure 2, regarding the values of the erythrogram.

The average number of erythrocytes (RBC) of pigeon chicks in group 2 is 23.88% higher than the average number of erythrocytes of pigeon chicks in the control group, the difference being statistically significant ($p<0.05$).

This observation means a strong intensification of hematopoiesis due to the compounds present in the composition of the dietary supplement. It results that the trace elements present in the composition of the used dietary supplement are responsible for the intense change of this parameter, a fact reported also in the literature (Constantin et al., 2004). Moreover, the composition of the food ration affects the blood count (Bălăceanu et al., 2017).

Table 2. The average values of the hemogram of the pigeon chicks from the 2 experimental groups

Hematological parameter	Group 1	Group 2
RBC (million/mm^3 blood)	3.35	4.15*
Hb (grams/dl blood)	11.2	12.6*
HTC (%)	37.3	38.1
MCV (fl)	113	93*
MCH (pg Hb/erythrocyte)	33.94	30.73
MCHC (g Hb/dl erythrocyte mass)	30.03	33.07

*$p<0.05$

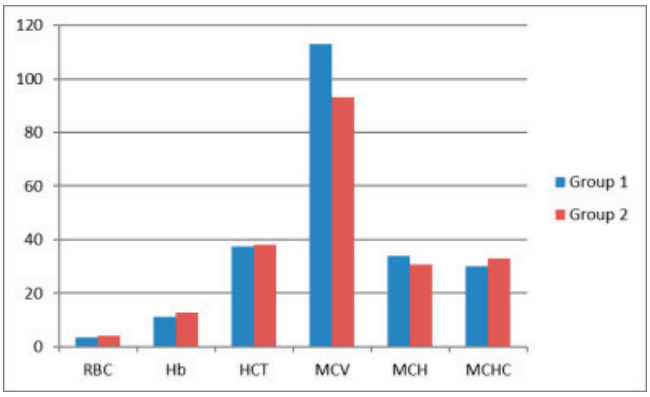

Figure 2. Erythrogram values in the case of the two experimental groups

The average number of erythrocytes (RBC) of pigeon chicks in group 2 is 23.88% higher than the average number of erythrocytes of pigeon chicks in the control group, the difference being statistically significant ($p<0.05$).
This observation means a strong intensification of hematopoiesis due to the compounds present in the composition of the dietary supplement. It results that the trace elements present in the composition of the used dietary supplement are responsible for the intense change of this parameter, a fact reported also in the literature (Constantin et al., 2004). Moreover, the composition of the food ration affects the blood count (Bălăceanu et al., 2017).

The average hemoglobin value (Hb) of the pigeons in group 2 is 12.5% higher than the average value of hemoglobin of the pigeons in the control group, the difference being statistically significant ($p<0.05$); it also indicates a strong intensification of hematopoiesis due to the substances present in the composition of the food supplement. The obtained results confirm that for the synthesis of hemoglobin (chromoprotein) both amino acids (for the synthesis of the protein component) and soluble iron (the nucleus of the hemoglobin molecule) are needed (Dojană et al., 2018).

The average value of hematocrit (HTC) in pigeon chicks in group 2 is 2.14% higher than the average value of hematocrit in pigeon chicks in the control group, difference which is not statistically significant. The obtained results confirm the data from the literature (Codreanu et al., 2019); it is known that this parameter is not changed by the intensity of hematopoiesis (the increase of the number of erythrocytes is accompanied by the increase of the amount of plasma, so that the percentage between the two major blood components remains relatively constant).

The average value of MCV (average corpuscular volume) in the pigeon chicks in group 2 is lower than the average value of MCV in the pigeon chicks in the control group by 17.7%, the difference being statistically significant ($p<0.05$). Comparing the value of the MCV parameter with RBC, it is found that there is a relationship of inverse proportionality between the intensity of erythropoiesis and the size of erythrocytes (the higher the number of erythrocytes is, the smaller their volume is).
Studying the literature (Evans et al., 2001) it can be noted that all MCV values obtained by us fall within the physiological limits (between 90 and 125 fl).

The average value of MCH (average corpuscular hemoglobin) in pigeon chicks from group 2 is 9.46% lower than the average MCH in pigeon chicks from group 1, difference which is not statistically significant. The decreases of this parameter compared to the control group can be explained by the intensification of hematopoiesis in the case of the experimental group, induced by the dietary supplement administered. However, it is observed that in the experimental group the amount of average erythrocyte hemoglobin

decreased, although the number of erythrocytes increased, which proves that the administered dietary supplement was not enough to support hemoglobin synthesis, so the hypochromia occurs. Comparing the data obtained by us with those presented in the literature (Gaytri et al., 1994; Codreanu, 2014), a slight hypochromia can be observed, the physiological values of the MCH parameter being between 33 and 45 pg hemoglobin/erythrocyte.

The average value of MCHC (average corpuscular hemoglobin concentration) in pigeon chicks from group 2 is 10.12% higher than the average value of MCHC in pigeon chicks from the control group, difference which is not statistically significant. The slight increase observed in the case of the experimental group may be due to the effect produced by the vitamins in the composition of the dietary supplement administered, knowing that some of them (B_{12}, C) stimulate hemoglobin synthesis (Dojană et al., 2018).
Regarding the comparison of the obtained values for this parameter, with the values communicated by other authors (Gaytri et al., 1994; Fudge, 2000; Evans et al., 2001), a decrease can be observed; it can be explained by not adapting the rate of hemoglobin synthesis to the intensity of hematopoiesis (erythrocyte formation).
As it was previously reported, the intensification of erythropoiesis is accompanied by a decrease in the volume of erythrocytes (MCV), but also by a decrease in the amount of hemoglobin contained in erythrocytes (MCH). In this context, it is normal for the MCHC parameter to decrease because it represents the amount of hemoglobin present in one dl of erythrocyte mass. So, if each erythrocyte contains less hemoglobin, it turns out that in one dl of erythrocyte mass, less hemoglobin will be found.

CONCLUSIONS

The administered dietary supplement determined the increase of the body weight of the pigeon chicks, the differences being significant compared to the control group, in all the experimental moments.

Regarding the effect of the dietary supplement on the erythrogram, significant differences were found compared to the control group for the following parameters: RBC and Hb (higher values) and MCV (lower values).

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ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

EFFECTS OF USING PROBIOTICS ON CALVES GROWTH RATES AND HEMATOLOGIC PROFILE

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Abstract

The aim of the current study was to evaluate the effects of using Enterococcus faecium as probiotic on dairy calves' growth rates and health status. During a 42 days trial, sixteen un-weaned Romanian Black Spotted calves were homogenously assigned in two groups: C (n = 8, control) and E (n = 8, treated with 2 x 10^8 CFU/ml of Enterococcus faecium). Body weight, blood sampling and diarrheic score were assessed at 0, 14, 28 and 42 days following administration of the probiotic. At the end of the trial, probiotic administration affected positively the experimental group, having a higher average daily gain with 23.96% compared to the control (ADG = 763.35g vs. 616.28g). Probiotic supplementation had no significant effects (P>0.05) on the hematologic profile and on calves health status. However, the prevalence of diarrhoea was higher in group C (32%) compared with group E (16%). Results suggest that throughout the use of Enterococcus faecium in un-weaned calves diets, the growth rates are improved and this could represent an approach in the control and prevention of enteric diseases.

Key words*: antibiotics, calves, health, probiotic, weaning.*

INTRODUCTION

In order to support dairy calves growth and development, nutrition and the health status are regarded as the main influencing factors (Malmuthuge et al., 2015). At the same time, dairy farmers express interest in accelerating the growth of calves (Liu et al., 2019), improving feed efficiency conversion (Turiello et al., 2020), while reducing the overall production costs (Xiao et al., 2020). Despite progresses reached in intensive rearing and feeding technologies, dairy calves' morbidity and mortality are still causing important economic losses (Raboisson et al., 2016). Moreover, un-weaned calf morbidity and mortality are frequently associated with enteric diarrhoea (Hulbert et al., 2016).

It was shown that induced stress factors such as isolation, ear-tagging, vaccination and weaning practices, have a significant influence on health and growth rates of calves, and the overall productivity (Mikus et al., 2020).

Nutrition and feeding strategies were shown to influence both growth and health of un-weaned dairy calves (Meale et al., 2016). In calves nutrition, antibiotics have been an important measure to balance health status and feed efficiency in intensive dairy production systems. Although both antibiotic feed additives and prevention antibiotics were banned in the EU starting year 2006, antibiotic traces were found in calves milk diets (Pogurschi et al., 2015; Chiesa et al., 2016). In the same time, avoiding bio-resistant microorganism induction and sustaining natural immune system (adapted or innate) could be the main key in controlling early bacterial infections (Van den Honert, 2019; Renaud et al., 2019; Flores et al., 2019).

Probiotics used as feed supplements are supported by the EU as potential alternatives to antibiotics (Regulation No. 767/2009). Among microorganisms with potential probiotic effects, *Enterococcus faecium* spp. were acknowledged as components of gastric

microbiome (Holzapfel et al., 2017), abundantly found in soil, water and waste (Aziz et al., 2019). In the last years, *Enterococcus faecium* spp. have gain interest for their beneficial probiotic attributes expressed in fermented foods (Marcondes et al., 2016; Brcina et al., 2019; Schittler et al., 2019), with a few strains being reported as bacteriocin producers (Qiao et al., 2019) and having significant antipathogenic traits (Hanchi et al., 2018). *Enterococcus faecium* NCIMB 11181 is used in pharmaceutical and para-pharmaceutical formulations, given that this strain targets pathogenic microorganisms, improving nutrient availability and the overall health status (Cangiano et al., 2020; Kayasaki et al., 2021).

The aim of the current study was to evaluate the effects of *Enterococcus faecium* NCIMB 11181 probiotic on un-weaned dairy calves growth rates, haematological profile and diarrhoeic prevalence.

MATERIALS AND METHODS

Sixteen un-weaned Romanian Black Spotted purebred calves, were homogenously assigned in two half-siblings' groups, balanced for sex, age and body weight during a 42 days trial, as follows:

- experimental group (E) nil per os (NPO) probiotic administration (n = 8);
- control group (C) with no probiotic treatment (n = 8).

Probiotic administration was performed daily during the first 28th days of the trial, with *per os* administration of 2 x 10^8 CFU/ml of *Enterococcus faecium* NCIMB 11181 (commercial probiotic strain). The following 14th monitoring days were used to observe probiotic post administration effects on calves development and haematological profile.

Calves were housed in individual hutches on deep straw bedding. During the first 3 days of life, calves were fed with 4 kg of colostrum per day, in two equal meals at 12 hours intervals. After colostrum administration, calves received a diet consisting out of 6 kg of milk substitute, into two equal meals per day [Eurolac 22/16, Schills, (125 g/L)]. Starting 10 days of life, the calves were offered unrestricted access to water, starter concentrates and alfalfa hay until the age of 3 months, when weaning took place.

Live body weight of calf was evaluated using a weighing scale platform, on the 0, 14th, 28th and 42nd days of experimentation.

Calves blood samples were collected on the 0, 14th, 28th and 42th day of the experiment. Samples from all calves were collected from the jugular vein, using vacutainer tubes with K_3EDTA (Kima®, Italy) containing 3.6 mg EDTA/K3 per ml of blood collected. Haematological determinations were done using a haematology analyser (Diatron, Abacus Junior Vet, Hungary). The hematologic screened parameters were: red blood cells count (RBC), red cell distribution width (RDW), haemoglobin concentration (HGB), mean corpuscular haemoglobin (MCH), platelets count (PLT), platelets percentage (MPV), platelets distribution width (PDW), total white blood cells count (WBC), lymphocytes count (LYM), monocytes count (MON), neutrophils count (NEU), haematocrits (HCT) and mean corpuscular volume (MCV).

During the 42 days of trial, diarrhoeic prevalence (PREV%) was registered daily. Diarrhoeic cases were reported and diagnosticated by the experimental farms' veterinarian.

Ethics statement

The research activities were performed in accordance with the European Union's Directive for animal experimentation (Directive 2010/63/EU). Use of animals and the procedures performed in this study were approved by the Scientific and Ethics Committee of the Research and Development Institute for Bovine Balotesti.

Statistical analysis

Statistical evaluation of live body weight and average daily gain were expressed as descriptive statistic, mean ± standard error of the mean. Although, calculation such as the prevalence rates were performed for the enteric cases. Hematological parameters were calculated using the analysis of variance (one-way ANOVA), at 0.05 level of significance.

Table 1. Chemical and amino-acid composition of calf starter concentrate and milk replacer

Chemical parameters	Units	Calf starter	Milk replacer
Nutritive units	UNL/kg	0.99	n.a.
Crude protein	%	18.5	22
Crude fat	%	1	16
Crude fibre	%	9	0.9
Methionine	%	0.36	n.a.
Lysine	%	0.9	n.a.
Calcium	%	2.69	n.a.
Phosphorus	%	0.69	n.a.
Salts	%	0.9	n.a.

RESULTS AND DISCUSSIONS

Calves live body weights are presented in Figure 1. At the beginning of the experimental trial, average live body weight ± SEM of calves were similar (C = 66.00 ± 7.27 kg and E = 66.96 ± 9.71 kg). Supplementing the calves diet with *E. faecium* had positive results, resulting in an overall average body weight higher with 7.9% in the experimental group (99.81 ± 9.74 kg), compared with the control group (92.50 ± 12.74). The increase in live body weight could be associated with antipathogenic specific attributes, enhancing the benefic bacteria populations and competitional pathogenic exclusion (Grigore et al., 2020). Our results are comparable with those of Sahu et al. (2019), which found a significant increase among calves body weights when using a solid *E. faecium* probiotic (70 mg/kg feed). High body weight gain shows that probiotic supplementation has positive effects, mainly based on enhancement of nutrient biodisponibility and benefic bacteria proliferation in the gastrointestinal microflora (Malmuthuge, 2017). Probiotic administration has proven positive effects after the first 14 days of administration, indicated by the body weight gain differences (2.81%) among control (73.43 ± 8.04 kg) and the experimental group (75.50 ± 10.19 kg). At 28 days, body weights were higher (3.37%) in the experimental group (86.94 ± 10.84 kg), compared with the control (84.10 ± 9.08 kg), indicating that long time-low concertation probiotic administration could support and enhance calves growth, in accordance with results published by Radzikowski (2017). In addition, important factors such as individuality, husbandry conditions and nutrition management are directly affecting calves performance (Irimia et al., 2020) and it is recommended to implement measures in order to support and promote calves health and development.

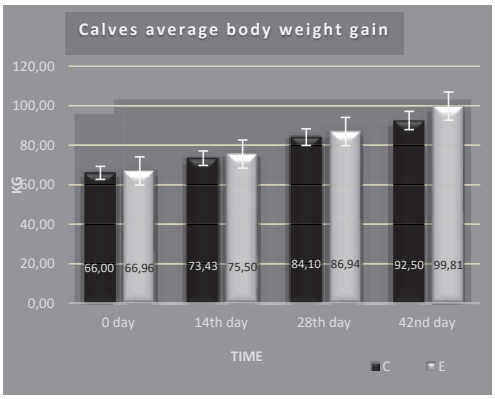

Figure 1. Calves average live body weight on days 0, 14, 28 and 42 of the experimental trial

Average daily gains are presented in Figure 2. At the end of the 42 days trial, probiotic administration affected positively the E group, having a higher average daily gain with 23.96% compared to the control (ADG = 763.35 g vs. 616.28 g). Our results show that probiotic administration in calves diets have positive effects (656.73 ± 78.78 g) starting the first 14th days of administration, leading to higher values with 14.98 g/head/day, compared with the control group (571.15 ± 62.76 g). In addition, Kelsey and Colpoys (2018) had similar findings when administrating a multi-strain probiotic containing *E. faecium* in calves diet, during the first 3 weeks of experimentation. On the contrary, Salazar et al., 2019 studied the *E. faecium* probiotic supplementation (70 mg/kg solid feed) and found no significant effects between control and the experimental group on average daily gain during the preweaning period. In addition, the same authors found that after weaning, calves treated with probiotic had lower body weights compared to their weaning weights, underlaying that probiotics had no residual effects on calves growth performance. Furthermore, Marcones et al. (2016) indicate no significant difference between pre-weaned Holstein calves growth performance fed with mixed prebiotics-probiotics and the control group.

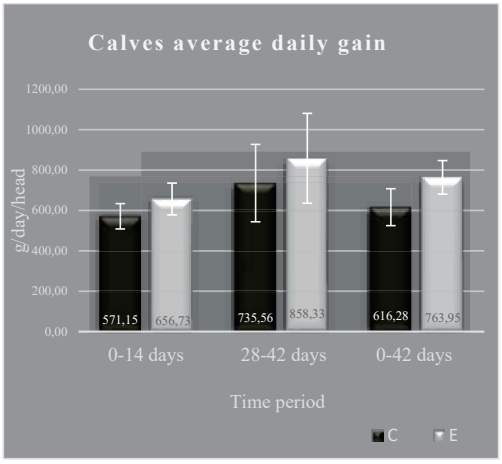

Figure 2. Calves average daily gain 0-14, 28-42 and 0-42 intervals of the experimental trial

Calves hematologic profile are displayed in Table 3. All haematological resulted data ranged within the reference values limits provided by Merck Veterinary Manual (2014) and Schalm's Veterinary Haematology (2011) (Table 2.). *E. faecium* dietary supplementation had no significant difference (P>0.05) between the control and experimental groups, indicating that probiotics might not interfere with the haemologic profile and could not modulate within leukocyte counts and leukocyte haematologic formula. RBC, HGB, HCT RDW, MCV and MCH were not influenced by dietary *E. faecium* supplementation (P>0.05), thus haematopoiesis related as red blood cells, haemoglobin and haematocrit could not be affected or stimulated by the probiotic treatment. Moreover, pre-weaned calves demonstrated a decreased activity of RBC before parasitic infestation (Emery et al., 2020) or stress challenges (Dahl et al., 2020). Low levels of RGB and HGB could be a natural response to anaemia, for example Fe^{+} or vitamin A deficiencies. However, calves raised on milk or milk replacer diets tend to present lower levels of RBC, as outlined by Weiss and Wardrop (2011). Platelets counts (PLT) and platelets haematologic formula (MPV, PDWc) were not influenced by *E. faecium* probiotic strain (P>0.05), indicating a physiologic and homeostatic health status without lesions, blood loss or abnormal cell encounter, in accordance with results by Vorobyeva and Medvedev (2020).

Table 2. Calves haematologic reference values intervals according to Merck Veterinary Manual and Schalm's Veterinary Haematology*

Haematologic parameter	Merck Veterinary Manual	Schalm's Veterinary Haematology
WBC	4-12x10^9/L	5.1-13.3 x10^3/µL
LYM	45-75%	1.8-8.1 x10^3/µL
MON	2-7%	0.1-0.7 x10^3/µL
NEU	15-45%	-
RBC	5-10 x 10^{12} g/L	4.9-7.5 x 10^6/µL
HGB	80-150 g/L	8.4-12 g/dL
HCT	24-46%	21-30%
MCV	40-60 fL	36-50%
MCH	-	14-19%
RDW	-	16-20%
PLT	-	160-650 x 10^3/L
MPV	-	4.6-7.4fL

*Merck Veterinary Manual 10th ed. and Schalm's Veterinary Haematology reference intervals. Table revised and inspirated after Weiss and Wardrop (2011) and Bedenicki et al. (2014)

Calves diarrhoea remains the main reason for calves morbidity and mortality (Urie et al., 2018). During our research trial, none of the calves died. At the beginning of the trial (first 14th days) the prevalence rate in the experimental group was similar to the control. The second period (the following 15th days) the prevalence rate was higher in the control group, compared with experimental group. The third interval (29-42 days) had a similar pattern, with none of the *E. faecium* supplemented calves developing the disease. The overall calves diarrhoeic prevalence was higher in the C group (32%), compared with the E group (16%). Results suggest that throughout the use of *E. faecium* in un-weaned calves diets, the diarrhoeic episodes are reduced, alongside with the severity of the symptoms, which could help in order to maintain the anti-bio resistance and to use the antibiotics only on special needs and severities. Besides growth promoting rates, *E. faecium* improved the immune response and this could represent an approach in the control and prevention of enteric diseases.

Table 3. Evaluation of probiotic effect on Romanian Black Spotted dairy calves haematologic profile between 0 and 42nd days of administration

Item		$\bar{x} \pm SEM$		p-value	Item		$\bar{x} \pm SEM$		p-value
		Control	Experimental				Control	Experimental	
WBC ($x10^9$/L)	0	9.52±0.82	10.14±1.35	0.73 (*N.S*)		28^{th}	27.89±0.92	27.08±0.74	0.51 (*N.S*)
	14^{th}	9.58±0.64	9.31±0.72	0.78 (*N.S*)		42^{nd}	26.98±1.03	24.47±0.89	0.09 (*N.S*)
	28^{th}	8.47±0.70	10.39±0.47	0.07 (*N.S*)	MCV	(fL) 0	32.75±0.92	32±1.38	0.66 (*N.S*)
	42^{nd}	9.73±0.85	9.93±0.47	0.83 (*N.S*)		14^{th}	32.5±0.65	31.25±0.65	0.20 (*N.S*)
LYM (%)	0	5.38±0.80	5.05±0.58	0.74 (*N.S*)		28^{th}	31.5±0.53	30.37±0.71	0.22 (*N.S*)
	14^{th}	5.64±0.54	5.45±0.38	0.78 (*N.S*)		42^{nd}	31.5±0.94	29.5±1.38	0.25 (*N.S*)
	28^{th}	5.58±0.40	5.97±0.62	0.58 (*N.S*)	MCH	(%) 0	11.63±0.53	10.8±0.38	0.22 (*N.S*)
	42^{nd}	5.47±0.48	6.15±0.17	0.31 (*N.S*)		14^{th}	11.04±0.21	10.38±0.27	0.07 (*N.S*)
MON (%)	0	0.178±0.028	0.187±0.055	0.90 (*N.S*)		28^{th}	10.7±0.23	10.13±0.28	0.14 (*N.S*)
	14^{th}	0.171±0.126	0.155±0.085	0.83 (*N.S*)		42^{nd}	11.1±0.74	10.55±0.65	0.58 (*N.S*)
	28^{th}	0.135±0.032	0.141±0.029	0.89 (*N.S*)	RDW(%)	0	23.73±0.56	25.26±0.90	0.17 (*N.S*)
	42^{nd}	0.191±0.155	0.183±0.069	0.93 (*N.S*)		14^{th}	24.58±0.53	25.08±0.76	0.59 (*N.S*)
NEU (%)	0	5.88±1.28	5.17±1.20	0.69 (*N.S*)		28^{th}	24.14±0.40	25.28±0.88	0.22 (*N.S*)
	14^{th}	3.71±0.19	3.70±0.66	0.99 (*N.S*)		42^{nd}	26.21±1.01	26.23±0.90	0.99 (*N.S*)
	28^{th}	3.06±0.37	4.60±0.76	0.13 (*N.S*)	PLT	($x10^9$/L) 0	415.63±37.56	398.86±43.51	0.77 (*N.S*)
	42^{nd}	4.87±0.94	5.12±0.90	0.85 (*N.S*)		14^{th}	439.38±31.93	460.88±39.46	0.68 (*N.S*)
RBC (10^{12}/L)	0	8.06±0.37	8.80±0.33	0.15 (*N.S*)		28^{th}	425.63±29.65	404.88±43.47	0.70 (*N.S*)
	14^{th}	8.48±0.30	8.88±0.30	0.35 (*N.S*)		42^{nd}	442±33.40	464.5±44.36	0.69 (*N.S*)
	28^{th}	8.85±0.29	8.95±0.33	0.82 (*N.S*)	MPV(fL)	0	5.69±0.17	5.49±1.70	0.42 (*N.S*)
	42^{nd}	8.65±0.40	8.11±0.48	0.39 (*N.S*)		14^{th}	5.56±0.09	5.46±0.12	0.50 (*N.S*)
HGB (g/L)	0	9.27±0.33	9.5±0.45	0.69 (*N.S*)		28^{th}	5.43±0.04	5.38±0.06	0.42 (*N.S*)
	14^{th}	9.35±0.33	9.21±0.33	0.77 (*N.S*)		42^{nd}	5.76±0.14	5.45±0.13	0.13 (*N.S*)
	28^{th}	9.43±0.26	9.15±0.22	0.44 (*N.S*)	PDWc	0	32.96±0.43	30.52±1.36	0.11 (*N.S*)
	42^{nd}	9.40±0.26	8.43±0.43	0.07 (*N.S*)		14^{th}	32.99±0.65	31.49±0.77	0.16 (*N.S*)
HCT (%)	0	26.28±0.91	28.19±1.51	0.29 (*N.S*)		28^{th}	31.98±0.31	30.71±0.72	0.13 (*N.S*)
	14^{th}	27.31±1.10	27.84±1	0.74 (*N.S*)		42^{nd}	31.86±1.55	31.18±0.82	0.70 (*N.S*)

Current results are in accordance with those of Renauld et al. (2019), which showed that by using multi-strain probiotics (including the *E. faecium* strain), the duration and the resolution of diarrhoeic episodes was reduced. Contrary, Smidkova and Cziek (2018) research suggests that *E. faecium* treatment of new born calves (+12h *post-partum* until 14th day of life) do not reduce the *E. coli* faecal counts.

CONCLUSIONS

The current study shows that supplementation of pre-weaned calves diets with *Enterococcus faecium* NCIMB 11181 probiotic strain could improve live body weight and average daily gain. At the same time, probiotic intervention might represent a viable control measure in order to mitigate enteritis symptoms and support calves health status recovery. More research is needed in order to elucidated the long-term effect of *E. faecium* on the calves growth rates, intestinal microbiota and health status.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

STUDY ON THE ACTION AND EFFICIENCY OF MIXES OF FOLIAR FERTILIZERS AND HERBICIDES IN THE WHEAT AND CORN CROPS

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Abstract

Liquid fertilizer, in the current period, represents an important share for agricultural crops. From the research of the herbicides used worldwide it is ascertained that in most of the cases mixes of products are used to obtain a wide action range. Usually mixes of herbicides and complementary action are used, thus obtaining products having a wide action range which enables their use in various fields. The use of the herbicide mixes has important technical and economic benefits: the number of crops treatments are significantly diminished, therefore of the equipment, manpower, thus obtaining important diminishment of the energy consumption.

Key words*: fertilizer, foliar, herbicide.*

INTRODUCTION

Taking into consideration the technical and economic benefits, we studied the possibility to obtain fertilizing compositions with herbicide mixes. In Romania, mixes made up of the acid 2.4D (2,4-dichlorophenoxyacetic acid) and Dicamba (3,6-dichloro-2-methoxybenzoic acid) manufactured and traded under the name of Ceredin Forte is homologated and licensed to be used by ALCHIMEX S.A. Specialists estimate that the assimilation and industrialization of liquid fertilizers constitutes one of the most important achievements in the field of fertilizers, due to the technical and economic advantages it presents (Akelah, 1996; Hall et al., 1994). These mixtures allow for a single operation to carry out a complex treatment: fertilisation, herbicide, insectification etc., which constitutes great technical and economic advantages (Horrigan et al., 2002; Matson et al., 1998; Mogul et al., 1996).

MATERIALS AND METHODS

To obtain a wide action range we used a mix of 2.4 D (28%) and Dicamba (35%) as dimethylamine (DMA). This systemic herbicide is absorbed by plants both by the root system and by the leaves (Muraviev et al., 1998).

The biochemical mechanism used by Ceredin to destroy the weed resides in deranging the growth processes and inhibiting the development of the root system.

CEREDIN FORTE is used to fight and control weeds in the corn, wheat, rye, barley, two row barley and oat crops (when the plant has 2-5 leaves). It is also used in the springtime during the twinning phase until the formation of the second node and the weeds are in the rosette phase (namely they have 2-6 leaves); consumption 2 l/ha for corn, wheat, barley; 1,5 l/ha for the two-row barley and barley.

Table 1. Main species of weeds destroyed by the CEREDIN type herbicide

Name of the weed		Control rate	
Common name	Scientific name	2,4-D	CEREDIN
Yarrow (milfoil)	*Archillea millefolium*	**	***
Corn cockle	*Agrosterma githago*	0	****
Mayweed / wild chamomile	*Anthemis* sp.	**	****
Wild bishop	*Biofora radians*	0	***
Heart-padded hoary-cress	*Cardania draba*	**	****
Shepherd's-purse	*Capsella bursza pastoris*	***	****
Creeping thistle	*Cirsium arvense*	***	****
Convolvulus	*Convulsis arvenis*	**	***
	Descurania sophia	***	****
Field (common) horsetail	*Equisentum arvense*	***	****
Cleavers	*Gallium aparine*	0	****
Chamomile	*Matricaria chamomilla*	0	****
Matricaria	*Matricaria inodora*	0	****
Corn poppy (corn rose)	*Papaver rhaeas*	0	***
black-bindweed	*Polygonum convulvulus*	**	****
pale persicaria	*Polygonum lapthiofolium*	**	****
Sheep's (red) sorrel	*Rumex acetosella*	**	***
Austrian fieldcress	*Rorippa austrica*	**	***
Corn Sow thistle, (Dindle, Field Sow Thistle, Gutweed, Swine Thistle)	*Sonchus arvensis*	**	****
	Sonchus oleraceans	**	***
Common chickweed	*Stellaria media*	0	***
Field Penny-cress	*Thalaspi arvense*	***	****
Corn (common) speedwell	*Veronica arvense*	0	****
Veronica	*Veronica hederifolia*	0	****
Common vetch	*Vicia angustifolia*	**	***
Tufted (cow) vetch	*Vicia cracca*	**	***
Hairy (tiny) vetch	*Vicia herusta*	**	***
Hungarian vetch	*Vicia pannonica*	**	***
Field pansy	*Viola arvensis*	**	***

LEGEND:
0 = inefficient; ** = the weeds are approximately 50% destroyed; *** = the weeds are approx. 75% destroyed; **** =the weeds are approx. 100% destroyed.

The CEREDIN FORTE herbicides are used to fight and control the weeds of the straw cereals; consumption 1 l/ha of crop.

In Table 1 there are the main species of weeds destroyed by the 2.4D acid and the CEREDIN type products (2.4-D and Dicamba).

From the data presented in Table 1 it clearly comes out that the CEREDIN products have a wider herbicide range than 2.4-D, therefore they were selected to make the mix of fertilizers and pesticides.

We may obtain concentrated emulsions of liquid foliar fertilizers and Ceredin, using emulsifier as thickening agents and dispersing agents. We decided that we may obtain compositions of chemical fertilizers as concentrated emulsion using liquid fertilizers (including foliar) and Ceredin, having the composition below:

- 100-129 g Ceredin;
- 300-350 g solution of foliar liquid fertilizers;
- 20-25 g emulsifier NF-10 (as thickening agent);
- 2.0-2.5 g polyvynilic polyvynil alcohol with GH = 88-92% (as dispersing agent).

To obtain a stable in time concentrated suspensions (in which no separations or sedimentations of products as sediment occur), to the obtained compositions we added various jellifying agents.

We used jellifying agents of the polysaccharide class and polyacrylamide solutions in concentrations of 0.1-0.5% compared to the total weight of the mix.

The Ceredin products are applied post-emergent, during the vegetation when the air temperature is of minimum 7°C, tending to be higher. The Ceredin type herbicides have a wide action range, therefore they are used to fight and control more than 200 species of annual and perennial dicotyledonous weeds, including those resistant to the action of the 2.4 D acid (in the mix two herbicides with complementary action are used, which determines a convenient widening of the action range). Liquid compositions of foliar fertilizers and Ceredin are used as concentrated suspensions. The suspension is made by inserting the Ceredin, the emulsifier and the dispersing agent into the fertilizer solution, by agitation, at 30-75°C. The obtained concentrated suspensions have been analyzed from the point of view of the stability of the active products (herbicides) they are made of. After 30-45 days from the making the diminution of the active products' (2.4 D acid and Dicamba) concentrations was no longer found.

As a conclusion, we may obtain concentrated suspensions of liquid fertilizers and Ceredin type fertilizers with appropriate physical-chemical stability in time. The solid mixes (as granules) made up of chemical fertilizers and Ceredin type herbicides were made by depositing the herbicides in solution on the fertilizers granules and eliminating humidity by means of a warm air current.

On the grounds of the theoretical and technical-economic reasons as well as of preliminary investigations, we reached the conclusion that liquid compositions of foliar fertilizers and Ceredin may be obtained as concentrated suspensions.

RESULTS AND DISCUSSIONS

I.C.P.P. (Research Institute of Corn Crops) tested the herbicide on experimental lots cultivated with Flamura variety wheat. The first treatment was carried out in April and the second one in May. The mix of herbicide fertilizer was sprayed using the manual pump. The observations were made 30 and 60 days after the treatment. The experiments were made in dryness conditions (high temperatures and absence of rain). As standard substance we used the Ceredin Forte herbicide. During the tests we also monitored the effect of the fertilizer on the way the plants develop and on the increase of the seeds production.

The results of testing the herbicide efficiency of the mix of fertilizer and Ceredin Forte are presented in Table 2.

The Cereals and Technical Plants Research Institute (I.C.C.P.T.) of Fundulea made the tests on the selection and efficiency (E) of post-emergent application of the mix of foliar liquid fertilizer and Ceredin Forte at a dose of 5.0 l/ha for the wheat crops (Hodosan, 2007). The treatments was carried out when the plants had 2-3 internodes and the dicotyledonous weeds had more than 4-6 leaves. To apply the mix we used 400 l of water/ha. The experiments were carried out in unfavorable weather conditions: prolonged dryness, high temperatures (35-41°C), extremely small quantity of rain.

The testing took place in the wheat field of Flamura 85 variety and the assessment of the herbicide efficiency was carried out 14 and 28 days after the treatment. Furthermore, the efficiency tests of the mix of fertilizer and Ceredin Forte in the corn crops were carried out by the scientific researchers within the Research Institute of Corn Crops (I.C.P.P.) of Bucharest. The experiments were made in conditions of high rate of weeds (the number of weds reached even 107 plants/m^2). On the grounds of the theoretic and technical – economic reasons as well as of preliminary investigations, we studied the possibility to realize compositions of foliar fertilizers and CEREDIN as concentrated suspensions.

The observations were made 30 and 60 days after the treatment.

The results of the tests of the herbicide efficiency of the mix of fertilizer and Ceredin Forte in fighting and controlling the weeds of the corn crops are presented in Table 4. Weeds represent a major threat to crop production (Ahmad, 2021; Gandini, 2020; Hodosan, 2007; Shavit et al., 1997).

The weeds present in the wheat crop (Table 3) when the treatment was made were:

- *Galium* (Gal.)
- *Papaver* (Pap.)
- *Anthemos* (Anth.)
- *Cirisum* (Cirs.)
- *Convulsuvus* (Conv.)
- *Veronica* (Ver.)
- *Delphinium* (Delph.)

Table 2. The herbicide efficiency of the mix of foliar fertilizer and Ceredin Forte in fighting the wheat crops weeds

Product	30 days after the treatment						60 days after the treatment					
	Dicotyledonous		Monocotyledonous		Total		Dicotyledonous		Monocotyledonous		Total	
	No. of plants/ m^2	E (%)	No. of plants/ m^2	E (%)	No. of plants/ m^2	E (%)	No. of plants/ m^2	E (%)	No. of plants/ m^2	E (%)	No. of plants/ m^2	E (%)
Untreated sample	10	-	8.5	-	18.5	-	8	-	5.5	-	13.5	-
Fertilizer + Ceredin Forte 4 l/ha	2	80	2	76.5	4	78.4	3	62.5	2	63.7	5	63
Ceredin Forte 1 l/ha (standard)	1	90	0	100	1	94.6	2	75	1.5	72.7	3.5	74.1

Table 3. Results of the herbicide activity efficiency f the mix of foliar fertilizer and Ceredin Forte in the wheat crops (ICCPT-Fundulea)

Product	Dose l/ha	Application cleaning	Efficiency		Average yield		Species of uncontrolled weed (according to the dominance)
			14 days	28 days	kg/ha	(%)	
Untreated	-	-	0	0	3200	100	Gal., Pap., Anth., Cirs., Conv., Ver., Delph.
Ceredin Forte (standard)	1.0	Post-treatment	90	90	3546	111	Conv., Gal., Delph.
Ceredin Forte+ Foliar fertilizer	5.0	Post-treatment	90	90	3520	110	Conv., Gal., Delph.

Table 4. The results of the herbicide action efficiency of the mix of fertilizer and Ceredin Forte in the corn crops

Product	30 days after the treatment						60 days after the treatment					
	Dicotyledonous		Monocotyledonous		Total		Dicotyledonous		Monocotyledonous		Total	
	No. of plants/ m^2	E (%)	No. of plants/ m^2	E (%)	No. of plants/ m^2	E (%)	No. of plants/ m^2	E (%)	No. of plants/ m^2	E (%)	No. of plants/ m^2	E (%)
Untreated sample	2.5	-	1.5	-	4	-	0.5	-	1	-	1.5	-
Un-weeded sample	17.5	-	80	-	97.5	-	21.5	-	88.5	-	107	-
Fertilizer + Ceredin Forte 4l/ha	0	100	27.2	66	27.2	72.2	5.5	74.5	22	74.3	27.5	74.3
Ceredin Forte 1l/ha	3.5	80	25	68.8	28.5	70.8	2.5	88.4	26	69.6	28.5	73.4

The weeds present in the corn crop when the treatment was made are mentioned below. As standard herbicide Ceredin Forte was used.

Annual dicotyledonous:

- *Amaranthus retroflexus*
- *Chenopodium album*
- *Galisonga parviflora*
- *Polygonum* spp.
- *Portulaca oleracea*
- *Solanum nigrum*
- *Sonchus oleraceus*

Perennial dicotyledonous:

- *Cirisium arvense*
- *Convolvulus arvensis*

Annual monocotyledonous:

- *Setaria* spp.
- *Echinochloa crus-galli*

The toxicity to mammals of the CEREDIN products is moderate, the average lethal doses (DL_{50}) being of 305-320 mg/kg of live weight. These herbicides pertain to the toxicity group III. DL_{50} for mammals of 2.4-D is of 350-360 mg/kg, the amine salt of 2.4 D has DL_{50}=980-1200 mg/kg (low toxicity). For mammals, Dicamba has DL_{50} = 1200-1300 mg/kg (low toxicity).

CONCLUSIONS

After the tests made at I.C.P.P. Bucharest, the following conclusions were drawn:
a) the mix of fertilizer-Ceredin Forte provides a satisfactory control of the wheat crops weeds;
b) the herbicides efficiency of the composition fertilizer-Ceredin Forte was comparable to the one of the substance used as standard (Ceredin Forte); the differences related to the herbicide efficiency of the mix of fertilizer-Ceredin Forte and the one recorded for the standard substance are in the limits of the specific errors of the statistic calculations;
c) in the evaluation of the tests results we must take into consideration the dryness conditions of the experiments (unfavorable: high temperatures, absence of rain) etc.;
d) because of the unfavorable weather conditions the data recorded on the effects of the fertilizers on the yield increase did not enable evaluations; yet the stimulating effects of the fertilizer on the plant development during vegetation were highlighted by a more intense coloration of the leaves representing the proof of the photosynthesis processes stimulation; moreover, we also noticed as a positive effect of the fertilizer, a higher resistance of the plants to the dryness.
As a conclusion, we assert that the mix of fertilizer and Ceredin Forte provides an adequate control of the weeds in the wheat crops.
After the tests carried out at I.C.C.P.T Fundulea, the following conditions were drawn:
a) the herbicide efficiency of the mix of foliar fertilizer and Ceredin Forte is satisfactory (88-90%), comparable to the efficiency of Ceredin Forte used as standard substance 14 days after the treatment and equal 28 days after (both products had a herbicide efficiency of 90%). As well, the selectivity of the mix fertilizer + herbicide was similar to the one of the standard product;
b) in the assessment of the herbicide efficiency we must take into account two determining factors:
b.1.) the treatment was far too late compared to the vegetative state of the weeds (the dicotyledonous weed had more than 4 – 6 leaves; the species *Convulus*, *Galium*, *Papver* and *Delphinium* were 10-15 cm tall; in this stage of weeds' vegetative development, the efficiency of the herbicides is significantly reduced;
b.2.) the assessment of the fertilizer's influence on the production of berries was not possible because of the dryness conditions of the experiments 28 days after the treatment, on the areas treated with mix of fertilizer Ceredin and those treated with Ceredin Forte (standard substance), the weeds totally dried out; given the circumstances, the yields increases for each separate case did not significantly differentiate (the treatment with the mix of fertilizer + herbicide recorded a 110% increase and for the lots treated with standard substance the yield increase was of 111%).
Using mixes of herbicides and fertilizers we obtain synergic effects between the components of those compositions, which is materialized in superior crops compared to the crops obtained when these products are separately used.
The mix of fertilizer – herbicide is very efficient on the mono and dicotyledonous weeds of the corn crops. As well, the toxicity of these mixes to mammals is moderate, pertaining to the toxicity group III.
For the corn crops, after the tests made within I.C.P.P. Bucharest, the following conclusions were drawn:
a) the efficiency of the herbicide action of the mix of fertilizer and Ceredin Forte was similar to the one of the product used as standard (Ceredin Forte); both the mix of fertilizer – herbicide as well as Ceredin Forte are highly efficient on the dicotyledonous and monocotyledonous weeds;
b) the assessment of the results of the herbicide efficiency of the mix of fertilizer with herbicide must take into consideration two important issues:
b.1.) the experiments were made in a period when certain weeds were in more advanced vegetative states, when they are more resistant to the herbicide action of Ceredin;
b.2.) the testing was carried out in conditions of extremely high weed rate (107 plants/m^2);
c) the conditions of the experiments (non irrigation) did not allow definite assertions related to the effects of the fertilizers on the yield increase.
d) Ceredin products have moderate toxicity in mammals

As an overall conclusion, we believe that the herbicide efficiency and selectivity of the mix of foliar fertilizer and Ceredin Forte were similar to those recorded when using Ceredin Forte herbicide as a standard substance for both the wheat crops and the corn one.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

PRELIMINARY STUDY OF DIETS EFFECTS ON PERFORMANCE, CO_2 EMISSION AND MICROCLIMATE VARIATION OF PRIMIPAROUS SOWS

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Abstract

At present, the widespread raising of swine in farms becomes a serious problem in terms of the emission of carbon dioxide (CO_2), having an environmental impact. The objective of this study was to investigate the effect of different diets on growth parameters changes in physical size, carbon dioxide (CO_2) emission and microclimate monitoring. For 28 days, 7 TOPIGS sows (average initial body weight, BW ~ 205.09 kg) were allotted in two dietary treatments: control (C diet, 1.6% soybean oil) and experimental (E diet), (with 1.6% hemp oil). The type of oil differed by their fatty acids (FA) composition. Sows were individually weighed, P2 backfat thickness was determined by ultrasound and morphometric measurements of body size were taken three times during lactation. The CO_2 emitted by sows and their litter was calculated. Microclimate parameters were recorded daily. The addition of 1.6% hemp oil in the diet improved sows final body weight and reduced weight loss ($P<0.05$). A high significant negative Pearson correlation was observed between PUFA intake, and their fraction (Σ n-3, Σ n-6, Linolenic and Linoleic as predominant n-3 and n-6 FA). The proportion of CO_2 exhalated by sows fed E diet was significantly lower compared to C diet. The CO_2 emission, temperature and relative humidity were affected ($P<0.001$) by the sows' physiological status. The results from this study are indicating that the hemp oil inclusion in the sow's diet has a beneficial effect on performance. Further studies are necessary to determine the nutrition impact on environmental conditions.

Key words*: carbon dioxide, morphometric measurements, performance, sows.*

INTRODUCTION

At present, continuing food-feed competition, land degradation and climate change represent significant sustainability challenges for the livestock industry. These changes have led to a progressive increase in the use of lower-cost co-products to replace partial maize and/or soybean meal, with emphasis on their role in swine diets (Wachenheim et al., 2006). The oils are one of the main alternatives argued by their functional property, for example, soybean oil and hemp seed oil.

As is well known, the most commonly used oil in the diet of sows is soybean oil, a valuable source of n-6 fatty acids. Hemp seed oil, compared to soybean oil is an ideal by-product rich in n-3 fatty acids, with an important function in development, health and immunity. However, in the literature, studies regarding utilization of hemp seed oil respectively hemp seed in feeding the sows are limited (Habeanu et al., 2018). Recent studies (Doreau et al., 2013; Habeanu et al., 2019; Habeanu et al., 2020) revealed that oil-rich ingredients used in pigs' diet could have a positive effect of reducing greenhouse gas emissions.

Over the past few years, environmental and welfare concerns in the livestock industry have increased. In the swine industry, air quality has become a sensitive subject these days, and have started to attract researcher attentions. Although various microclimate installations are currently on the market and are being promoted, indoor air quality is still a challenge to farmers.

Poor indoor air quality can have an impact on swine health, productivity and well-being, besides being a health risk to farm workers.

Furthermore, studies have revealed that poor indoor air quality is often associated with respiratory diseases, stress and decreased pig productivity. (Cleveland-Nielsen et al., 2002; Michiels et al., 2015; Roque et al., 2018).

Livestock production is one of the most important sources of greenhouse gases emissions (Dong et al., 2009). The most fixed gases registered in swine facilities is carbon dioxide (CO2) which play a major role in indoor air quality measurements. The two major sources of CO2 production in pig housing are animal respiration and manure release (Habeanu et al., 2020).

The objectives of this paper consist in: i) assess the effect of dietary hemp seed oil relative to soybean oil in lactating sows' diets on performances; ii) evaluate the CO_2 emitted by sows and their piglets; iii) measuring of changes of indoor microclimatic parameters measurements.

MATERIALS AND METHODS

The present experiment was conducted at the experimental farm of INCDBNA Balotesti, according to Law 43/2014/Romania. All the experimental procedures were approved by the Ethical Committee (Protocol no. 699/2020).

Animals and Diets

A total of 7 TOPIGS primiparous hybrid sows were tested in this biological trial.

On day 105 of gestation, sows were moved to a farrowing room and kept in individual farrowing crates. Immediately after farrowing, the sows were divided into 2 experimental groups (Table 1), and feed with different diets: control (C; 1.6% soybean oil) and experimental (E; 1.6% hemp oil). The variable was the type of oils with different fatty acids (FA) composition summarized in Table 2.

Table 1. Ingredients and nutrient composition of sows' diet during gestation and lactation

Ingredients, %	**Lactation**	
	C	**E**
Corn	56.87	56.87
Rice meal	10.00	10.00
Soybean meal	18.00	18.00
Sunflower meal	10.00	10.00
Soybean oil	1.60	-
Hemp oil	-	1.60
Lysine	0.02	0.02
Calcium carbonate	1.75	1.75
Monocalcium phosphate	0.15	0.15
Salt	0.40	0.40
Choline premix	0.20	0.20
Vitamin-mineral premix P5+6	1.00	1.00
Phytase	0.01	0.01
Calculated chemichal composition, %		
DM	89.71	89.70
Metabolisable energy (MJ/kg)	3070	3070
Crude protein	17.97	17.42
Lysine	0.87	0.87
Digestible Lysine	0.69	0.69
Methionine + Cystine	0.65	0.65
Digestible Methionine + Cystine	0.52	0.52
Calcium	0.92	0.93
Phosphorus	0.80	0.81
Crude fiber	5.59	5.91
Crude fat	5.62	5.24

ME and amino acid contents were calculated based on feed composition. Gestation diets were provided in one meal/day; lactation diets were provided in two meals/day.

Vitamin mineral premix P5 + 6: 9000 IU vitamin A; 1500 IU vitamin D3; 50 IU vitamin E; 2 mg vitamin K3; 1.5 mg vitamin B1; 5.2 mg vitamin B2; 15 mg vitamin B3; 8.1 mg vitamin B5; 2 mg vitamin B6; 0.10 mg vitamin B7; 0.5 mg vitamin B9; 0.03 mg vitamin B12; 39 mg of Mn; 100 mg of Fe; 15 mg Cu; 100 mg Zn; 0.3 mg I; 0.22 mg Se; 0.25 mg Co; 60 mg antioxidant.

The experimental diet contained a higher amount (>3.42 times) of Σ n-3 FA and a ratio of 3.53 times greater in E diet n-6: n-3. Throughout the entire experimental period, sows had *ad libitum* access to feed and leftovers were registered daily. The sows had free access to the water both in the individual pens.

Table 2. Fatty acids profile of soybean oil, hemp seed oil and diets used during the experiment

Fatty acids, % of total FAME	Oils		Diet	
	Soybean oil	Hemp seed oil	C	E
Σ SAF	14.95	12.86	15.76	16.66
Σ MUFA	27.22	14.59	33.05	29.86
Σ PUFA	57.75	72.58	51.18	53.46
Σ n-3	6.58	17.30	1.54	5.27
Linolenic	6.68	17.06	1.03	5.07
Σ n-6	52.29	53.79	49.64	48.19
Linoleic	51.11	55.28	49.60	48.12
n-6/n-3 ratio	7.77	3.20	32.23	9.14

SFA - saturated FA; PUFA - polyunsaturated FA; MUFA - monounsaturated FA.
Total SFA: C8:0 + C10:0 + C12:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C24:0; Total MUFA: C15:1 + C16:1 + 17:1 + Total trans 18:1+ C18:1 cis-9 + C18:1 cis 7 + C20:1n-9 + C22:1n-9 + C24:1n-9; Total PUFA: C18:2n-6 + C18:3n-6 + C18:3n-3 + C18:4 n-3 + CLA + C20:52n-6 + C20:3n-3 + C20:4n-6 + C22:2n-6 + C20:5n-3 + C22:3n-6 + C22: 3n-3 + C22:4 n-6 + C22:5 n-3 + C22:6n-3.

Measurements

All measurements of sows (body weight (BW), backfat, morphometric) were made after farrowing and at weaning.

Sows backfat thickness (mm), lean meat thicknesses (mm) and loin muscle percentage (%) was measured between 3rd and 4th lumbar vertebra at 7 cm, laterally (P2 position), from the backline using a PIGLOG 105 (SFK Technology, Denmark) ultrasound scanner. The final result for backfat thickness was the average value from both sides of P2 position measurements. Before measurements age (days) and live weight (kg) of sows were introduced into the device.

The morphometric measurements of the sows (heart girth, length and flank) were made by using a cloth tape measure. Heart girth is defined as the circumference of the sow just behind the forelegs and shoulders and in front of the first mammary gland, while flank-to-flank is taken from the bottom of the left flank to the bottom of the right flank, running over the top of the sow. The morphometric and backfat measurements of sows were taken when the sows were upright and relaxed.

Indoor microclimatic measurements such as carbon dioxide (CO_2), relative humidity (% rH), atmospheric pressure (hPa), air temperature (°C) and room airflow speed were continuously measured during the lactation phase at 8:00, 10:00 and 14:00 hours with a portable digital instrument Testo. As a final result, the average of the three measurements was taken into consideration.

Chemical composition

Samples from C and E diets were taken at the beginning of the experiment and were chemically analysed for weende by standardised methods (Commission Regulation (EC) no. 152, 2009) and fatty acids.

Fatty acids. The fatty acids profile was determined by gas chromatography (Perkin Elmer Clarus 500 gas chromatograph, Massachusetts, United States), fitted with flame-ionization detector (260°C temperature) and capillary separation column with high polar stationary phase Agilent J & WGC Columns, (United States), DB-23 dimensions 60 m x 0.250 mm x 0.25 µm. The FA were identified by comparison with blank chromatograms and were subsequently determined quantitatively as a percent of total FAME. SUPELCO 37 component FAME Mix was used; 10 mg/ml as a standard solution of methylated FAs and also Soybean Oil and Sunflower Oil; SUPELCO, as reference material was used. We used hydrogen as carrier gas and oxygen as burning gas, (method described by Hăbeanu et al., 2016). The mean values for FA composition were presented as g FA/100 g total FA ester methyl (%), and g/kg diet.

Statistical analysis

The data were submitted to variance analysis using the General Linear Model (GLM) of the SPSS program (SPPS, 2011). The results were expressed as mean values and standard error of the mean (SEM). To evaluate the relationship between certain parameters, Pearson's correlation was used. Differences were considered significant if $P<0.05$, and highly significant when $P<0.001$.

CO_2 emitted was calculated from the heat production (HP, MJ/day, corrected for density (22.4 l/mol) and molar weight (44 g/mol) using the formula described by Rigolot et al. (2010)

and Noblet (1987) adapted by Habeanu et al. (2020) adapted for sows and piglets.

RESULTS AND DISCUSSIONS

Growth parameters

As known sows' diet supplementation with oils during lactation has been shown to improve milk composition as well as piglet's performance. However, studies of the benefits of hemp seed oil inclusion in lactating sows diets, on body performances it is limited.

In order to prevent the excessive gain of body condition the average feed intake during gestation was limited to 2.80 kg/d.

During lactation, the sows fed C diet registered an average feed intake of 5.82 kg/d while the E group consumed an average feed intake of 6.09 kg/d, without significant differences between groups.

Table 3. Means of feed, calculated fatty acids intake and performance

Item**	C	E	SEM	P-value*
Feed intake kg/d	5.82	6.09	0.07	*0.108*
Fatty acids intake g/kg feed				
Σ SAF	41.90	41.94	0.59	*0.973*
Σ MUFA	65.92	57.27	0.93	*0.0001*
Σ PUFA	125.88	145.69	2.04	*0.0001*
Σ n-3	8.93	19.35	0.41	*0.0001*
Linolenic	8.99	19.10	0.40	*0.001*
Σ n-6	117.49	125.86	1.75	*0.018*
Linoleic	116.22	124.24	1.73	*0.021*
n-6/n-3 ratio	61.50	59.99	0.86	*0.388*
Growth parameters				
Sows BW -12 h after farrowing	203.00	205.50	0.79	*0.102*
Sows BW -Weaning (28d)	176.67	186.25	1.12	*0.0001*
Total body weight loss	*-26.33*	*-19.25*	*1.16*	*0.001*
Total no. of piglets borne alive, head	14.67	10.50	1.51	*0.139*
Piglets BW, kg	1.30	1.62	0.09	*0.001*
ADG, g	237.19	247.79	1.72	*0.002*
No dead piglets	1.00	0.5	0.36	*0.542*

[1]C: control group; [2]E: experimental group; [3]SEM: standard error of the mean.
*P < 0.001 highly significant difference; P < 0.05 significant difference; P < 0.10 tendency of influence; P > 0.10 not significant.
SFA - saturated FA; PUFA - polyunsaturated FA; MUFA - monounsaturated FA.
Total SFA: C8:0 + C10:0 + C12:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C24:0; Total MUFA: C15:1 + C16:1 + 17:1 + Total trans 18:1 + C18:1cis-9 + C18:1 cis 7 + C20:1n-9 + C22:1n-9 + C24:1n-9; Total PUFA: C18:2n-6 + C18:3n-6 + C18:3n-3 + C18:4 n-3 + CLA + C20:2n-6 + C20:3n-3 + C20:4n-6 + C22:2n-6 + C20:5n-3 + C22:3n-6 +C22:3n-3 + C22:4n-6 + C22:5n-3 + C22:6n-3.

The average of estimated FA intake (Table 3) differed (P=0.0001) between diets. Thus, sows fed E diet ingested a lower amount of monounsaturated FA (<13.12%; MUFA) and a higher amount of polyunsaturated FA (>15.74%; PUFA), n-3 (>53.85%), respectively n-6 (>7.12%) compared with sows fed C diet.

The average BW loss at weaning was lower (<5.42%; P = 0.0001) in the sows fed E diet compared with sows fed C diet. The total weight loss of sows, from farrowing to weaning were significantly affected, Contrary to our results Vodolazska and Lauridsen (2020) observed that sows' BW ranging from 320 ± 10.8 kg (hemp seed oil diet) to 346 ± 11.4 kg (soy-bean oil diet). Lows et al. (2018) that diet supplementation with palm oil has not affected sow performance.

According to Lawlor and Lynch (2007), Cozzanet et al. (2018) and Habeanu et al. (2018) sows body weight loss during lactation occurs as a result of high milk yield and a relatively small appetite. Different authors suggest that sows can lose between 15-40 kg of body weight during lactation (Hansen, 2013; Cools et al., 2014).

The main effects of diet and period on sows' body measurements are outlined in Table 4.

Lactating sows feed E diet had a backfat thickness lower 25.88% (P = 0.011) compared to lactating sows fed C diet while lean meat registered a value 7.25% higher (P = 0.009);

compared to a similar backfat thickness evolution on lactating sows has been noticed also by Eissen et al. (2003). However, Lavery et al (2019) using salmon oil, mentioned that sow body condition was not improved and back-fat depth in his study decreased as lactation progress, not as dietary treatment.

Table 4. Effect of hemp oil on sows' body measurements during lactation

Diets	Period	Backfat thickness (mm)	Loin eye (mm)	Lean Meat (%)	Heart grind	Flank	Length
C	AF	18.33	44.67	52.07	133.67	148.67	133.00
	d-5	17.50	41.00	52.33	135.67	147.67	136.33
	d-21	14.17	40.33	55.50	126.67	141.67	127.00
	d-28	11.67	42.00	58.03	125.33	134.33	132.67
E	AF	13.25	43.25	56.80	133.25	147.50	137.75
	d-5	14.75	49.25	56.05	131.75	144.75	136.25
	d-21	10.50	43.50	59.33	128.00	141.00	135.75
	d-28	10.50	43.75	59.38	125.75	128.75	137.50
SEM		0.71	1.03	0.71	1.10	1.87	1.59
Main effects							
Diet							
C		15.42	44.36	55.01	131.11	140.28	132.00
E		12.25	47.83	58.09	130.08	137.04	135.17
Period							
	AF	15.79	43.96	54.43	133.46	148.08	135.38
	d-5	16.13	45.13	54.19	133.71	146.21	136.29
	d-21	12.33	41.92	57.41	127.33	141.33	131.38
	d-28	11.08	42.88	58.70	125.54	164.21	135.08
P-value							
Diet		*0.011*	*0.181*	*0.009*	*0.749*	*0.425*	*0.198*
Period		*0.010*	*0.737*	*0.033*	*0.015*	*0.007*	*0.755*
Diet*Period		*0.670*	*0.458*	*0.773*	*0.803*	*0.946*	*0.841*

C: control group, [2]E: experimental group. AF: after farrowing; d-5: day 5; d-21: day 21; d-28: day 28
SEM: standard error of the mean.
*P < 0.001 highly significant difference; P < 0.05 significant difference; P < 0.10 tendency of influence; P > 0.10 not significant.

During the experimental period, the significant difference was noticed for all parameters evaluated. Although 5d AF the backfat thickness increased slightly (2.15%), up to 28d the backfat thickness decreased 29.8% (P<0.01), respectively. A contrary tendency was observed with respect to lean meat which registered a higher value at 28d AF compared to first d (7.8%, P<0.033). A backfat reduction in sows, AF was also observed by Eissen et al. (2003), Cozzanet et al. (2018) and Lavery et al (2019). According to Song et al. (2010) and the authors mentioned above, lactating sows use more energy for milk production which automatically leads to a reduced backfat, body weight losses or other negative productive performances.

The sampling time had a significant effect on heart grind and flank measurements (P = 0.015 respective P = 0.07).

There was no interaction found between diet and period for any of the body measurements determined on sows.

CO_2 emitted and microclimatic condition

The indoor animal husbandry environments are one of the most important in the swine sector because have a direct impact on production efficiency respectively animal health and welfare. As well-known carbon dioxide is the second most important greenhouses gas emissions that can be generated during the production due to the heating, ventilation, feeding, manure handling, and washing (Hörndahl, 2008, MacLeod et al., 2013 Boontiam et al., 2015).

The calculated sows' and their litter CO_2 emissions and the registered mean values of the microclimate in the sows' farrowing unit are presented in Table 5.

In HP calculation was used EN and EM intake. In our study EN and EM intake was similar between diets and did not differ significant.

Our data revealed that the proportion of CO_2 exhalated by sows fed E diet was significantly lower, which can be attributed to the fact that HP value was also decreased compared to C diet. A possible explanation can be attributed to the highly significant negative correlation between PUFA, especially n-3 FA in which linolenic FA is predominant and CO_2 emitted by animal's respiration.

Table 5. Heat production and CO_2 exhalation from sows and piglets

Item	C	E	SEM	P-value
Sows				
EN intake	50.14	52.52	0.73	*0.108*
EM intake	67.28	70.48	0.98	*0.108*
HP (MJ/day)	21.29	20.51	0.10	*0.0001*
CO_2 emitted (kg/d)	1893.34	1823.74	9.16	*0.0001*
Piglets				
HP (kcal/day)	433.27	449.60	2.66	*0.002*
CO_2 emitted (kg/d)	37.99	39.43	0.23	*0.002*
TOTAL CO_2 Emitted kg/d	1931.33	1863.17	9.12	*0.0001*

C: control group; E: experimental group; EN: net energy; EM: metabolizable energy; HP: heat production;
SEM: standard error of the mean.
P < 0.001 highly significant difference; P < 0.05 significant difference; P < 0.10 tendency of influence; P > 0.10 not significant.

Regarding the CO_2 emitted by piglets we observed that piglets from sows fed E diet registered a higher HP and CO_2 production compared to C diet (> 3.79%, P = 0.002). This was expected since there is a strong correlation between CO_2 production and body weight (r = 0.83). Thus, animal growth necessitate energy, which automatically will lead to an increase CO_2 production.

According to Forcada and Abecia (2018) and CIGR (2002) the estimated production of respiratory CO_2, on the basis of body weight and feed energy intake, is 2.23-3.68 kg CO_2 per head for gestating and lactating sows respectively 0.88 kg CO_2 per head for weaned piglets.

The correlation between dietary FA and production CO_2 from sows and piglets is presented in Table 6. As can be observed all correlation were negatively between CO_2 and FA.

As presented in Table 7, we can observe that the amount of CO_2 emissions registered in the farrowing unite it is relative higher. Part of the CO_2 concentration is from animal respiration and part of the manure.

Compared to our values, Stinn et al. (2014) recorded higher CO_2 concentration 1556 (± 783) ppm, 1631 (± 811) ppm, and 1594 (± 797) ppm for the farrowing room, and mentioned that only CO_2 continued to increase, with the age of piglets while N_2O and CH_4 remained unchanged.

Table 6. Pearson corelation between calculated CO_2 emissions and fatty acid intake

Items	CO_2 emitted			
	Sows		Piglets	
	r	P	r	P
Σ SAF	-0.40**	*0.0001*	0.03	*0.636*
Σ MUFA	-0.29**	*0.0001*	-0.04**	*0.616*
Σ PUFA	-0.47**	*0.0001*	0.10	*0.155*
Σ n-3	-0.43**	*0.0001*	0.19	*0.005*
Linolenic	-0.43**	*0.0001*	0.19**	*0.006*
Σ n-6	-0.44**	*0.0001*	0.07	*0.345*
Linoleic	-0.44**	*0.0001*	0.06	*0.352*
n-6/n-3 ratio	-0.39**	*0.0001*	0.2	*0.772*

**Corelation is significant at the 0.01 level (2-taild); * Corelation is significant at the 0.05 level (2-taild)

Table 7. Physical microclimatic parameters in the farrowing unit

Items	MEAN	Minimum/ maximum	SD
CO^2 (ppm)	1264.7	798.8/3952.7	102.5
hPA	1004.03	986.5/1016.4	0.82
Temperature (°C)	24.6	21.9/25.5	0.1
Relative humidity (%)	65.4	44/99.9	1.64
Airflow velocity (m/s)	0.1	0.0/0.28	0.01

According to Philippe and Nicks (2014), farrowing sows, including piglets are associated with the highest CO_2 emissions, as a consequence of *ad libitum* feeding and intensive productive status (milk production and growth). Another cause for the increased CO_2, can be attributed to the temperature. There are several studies (Groenestein et al., 2003; Moehn et al., 2004; Philippe and Nicks, 2014) demonstrating that the level of CO_2 emission doubled when temperature increased from 15 to 20°C.

As a result of extensive temperature, ventilation and animal activity effects, same authors (Groenestein et al., 2003; Moehn et al., 2004;

Philippe and Nicks, 2014) observed a diurnal pattern of gassed emissions from pig houses. Thus, the highest gas emissions were registered during feeding time.

The registered mean values of temperature were of 24.6°C. An increase of temperature values was expected during lactation period, because the lower critical temperature for piglets in the first days of life is around 33°C (Bloemhofet al., 2008).

The relative humidity values recorded in our study are in agreement with values obtained by Romanini et al. (2008) and Justino et al. (2014) that registered a relative humidity of 75% respective 69.8%. According to Justino et al. (2014), the relative humidity optimum value for pigs is between 60 to 80%. Regarding the atmospheric pressure and the air flow velocity, this were within recommended parameters.

CONCLUSIONS

The addition of hemp seed oil in the sows lactating diet had a beneficial result and have proven to minimise weight loss after farrowing. A high significant negative Pearson correlation was observed between PUFA intake, and their fraction (Σ n-3, Σ n-6, Linolenic and Linoleic as predominant n-3 and n-6 FA). The indoor microclimate from farrowing unit was within the normal parameters and did not affect the lactating sows.

ACKNOWLEDGEMENTS

This study work was carried out with the support of the Ministry of Agriculture and Rural Development of Romania through Sectorial project ADER 9.1.4./2019.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

EFFECT OF THE PROBIOTIC BAYKAL EM-1 ON THE GROWTH PERFORMANCE, BLOOD PARAMETERS AND BEHAVIOR OF WEANED PIGS

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Abstract

The aim of the study was to investigate the effect of the probiotic preparation Baykal EM-1 on the growth performance, blood parameters and behaviour of weaned pigs. A research experiment with a total of 96 growing pigs, divided into two groups - control (48) and experimental (48), fed with the probiotic Baykal EM-1 in the amount of 10 ml/kg feed, was carried out. The indicators average live weight, feed consumption, daily gain, feed conversion, blood parameters and behaviour of pigs, were studied. The following conclusions were made: The addition of Baykal EM-1 (10 ml/kg feed) in weaned pig diets improved the average daily gain by 11% (P = 0.031). The higher number of leukocytes and lymphocytes in pigs from the experimental group (P<0.001), compared to the control group, may be an indicator of better health and higher immunity. A trend for better comfort in animals with the microbiological supplement in the feed, contributing to the better absorption of nutrients, has been established. The results obtained in this study show that the combination of probiotics Baykal EM-1 has the potential for use as a dietary supplement in weaned pigs.

Key words*: behaviour, blood, pigs, probiotics, weight.*

INTRODUCTION

The ban on the use of antibiotics in pig diets as growth promotors was introduced on 1 January 2006 due to concerns about antibiotic residues in food products of animal origin and the development of bacterial resistance (Pluske et al., 2002). The search for alternative strategies to improve animal production and health has enforced the use of probiotics in animal husbandry (Gu et al., 2006; Wang et al., 2009). The main reason for their application is the achievement of some beneficial effects, such as maintaining the balance of the intestinal microbiota and effectiveness in the fight against pathogens to both animals and consumers (Markowiak & Śliżewska, 2018). Probiotics have the ability to trigger the activity of the immune system and increase the body's resistance to diseases (Chomakov et al., 1990; Vieco-Saiz et al., 2019). They directly affect the intestinal microflora, the secretion of enzymes and their activity. This improves the functions of the digestive tract and improves metabolism.

A well-balanced intestinal microflora is able to affect the integrity of the intestinal barrier against colonization by pathogens through its metabolic function and thus stimulates the immune system to overcome inflammation. The physiological and psychological stress during the weaning period of the pigs - separation from the mother, regrouping, change in diet, etc. (Lalles et al., 2007), compromises the intestinal microbiota and leads to intestinal dysfunction. The use of probiotics to restore intestinal microbial balance is particularly relevant at this time (Ahmed et al., 2014). In recent years, numerous studies advocate probiotics provide improved nutrient utilization, protection against pathogens, and increased productivity in the pig industry, have been published (Chaucheyras-Durand & Durand, 2010; Ezema, 2013; Devi & Kim, 2014). The aim of the current study was to establish the effect of Baykal EM-1 on the productivity, blood parameters and behaviour in growing pigs.

MATERIALS AND METHODS

The experiment was carried out at the State Enterprise Experimental Farm at Agricultural Institute, Shumen, Bulgaria. A total of 96 weaned pigs from the Danube white breed, divided into two groups - control (48) and

experimental (48), were used. Each group consisted of 6 pens with 8 pigs in a pen. The equalization of the animals was done by origin, age, live weight and sex (equal number of males and females in one pen). The initial average live weight of the pigs was 9.7-9.8 kg. The experiment lasted for 39 days after weaning the pigs until reaching 24-25 kg live weight. Both groups of pigs were fed *at libitum* and received standard feed for the category (NRC, 2012). The contents of the diet and its composition are shown in Table 1.

Table 1. Component composition and analysis of compound feed for weaned pigs

Components	**%**	**kg**
Maize	25.25	252,500
Barley	10.00	100,000
Wheat	27.00	270,000
Bioconcentrate – 12[a]	29.60	296,000
Wheat bran	8.00	80,000
Synthetic lysine, 98%	0.15	1,500
Total:	100.00	1000,000
One kg compound feed contains:		
Metabolizable energy, kcal	3017	
Crude protein, g	18.5	
Lysine, g	1.00	
Methionine + cystine, g	0.63	
Threonine, g	0.68	
Tryptophan, g	0.23	
Crude fats, g	2.52	
Crude fibres, g	5.15	
Calcium, g	1.00	
Phosphorus, g	0.53	

Legend:
[a]The bio-concentrate BC12 contents: 39.90% Crude protein, 1.05% Crude fats, 5.70% Crude fibres, 14.70% Crude ash, 2.72% Calcium, 1.22% Phosphorus, 0.90% Digestible phosphorus, 1.00% Threonine, 1.93% Lysine, 0.86% Methionine, 1.54% Methionine + cystine, 0.40 mg/kg Sodium, 465.00 mg/kg Zinc oxide, 190.00 mg/kg Iron sulphate, 165.00 mg/kg Manganese oxide, 85.00 mg/kg copper sulphate, 1.30 mg/kg Sodium selenite, 3.65 mg/kg Calcium iodate, 40000 UI/kg Vitamin A /retinyl acetate/, 6000 UI/kg Vitamin D3 / cholecalciferol /, 320 mg/kg Vitamin E, 40 mg/kg Antioxidants.

Water was provided by nipple drinkers, one for each pen. The microbiological preparation Baykal EM-1 (10 ml/kg of feed) was added to the feed of pigs from the experimental group. The probiotic Baykal EM-1 is a collection of bacterial cells and metabolic products of the bacteria *Lactobacillus casei* 21, *Lactococcus lactis* 47, *Saccharomyces cerevisiae* 76 and *Photopseudomonas palistris* 108, in the form of a clear liquid without sediment with light-to-dark brown colour, pH 2.8-3.5, and a pleasant smell of kefir silage.

During the experiment, the growth performance indicators of pigs were monitored. Live weight was recorded individually, and all pigs were weighed at the beginning and at the end of the experiment.

Feed consumption was recorded daily, on a pen level, by weighing the feed immediately before feeding. Any residues were removed and weighed from the feeders (if feed left) and subtracted from the quantity of the feed for the previous day. The average daily gain and the average feed conversion were calculated. In order to identify possible differences in pig comfort between the two groups, the behaviour of pigs was monitored on two consecutive days for 24 hours at the beginning and at the end of for the previous day. The average daily gain and the average feed conversion were calculated.

In order to identify possible differences in pig comfort between the two groups, the behaviour of pigs was monitored on two consecutive days for 24 hours at the beginning and at the end of the experiment. Monitoring of behaviour was carried out by video cameras, which were located on the ceiling above the pens and covered four pens in each of the two treatments (Fig. 1). The video recordings were analysed every three minutes, taking into account the behavioural reactions such as movement, lying down and eating.

Figure 1. Observation of behaviour in growing pigs through video observations

At the end of the experiment, blood samples were taken from the eye sinuses from 10 pigs from each groups and were tested for differences in blood parameters in a laboratory with specialized pig kits. The full blood count indicators were analysed with the Diatron Abacus 5 Haematology Analyzer and methods as follows:

- Leukocytes - by conductometric and visual optical method;
- Differential blood count - laser MAPSS technology;
- Hemoglobin - by cyan-methaemoglobin method;
- Erythrocytes and MCV - by conductometric method;
- Erythrocyte indices – calculated;
- Hematocrit - by an indirect method based on conductometric methods;
- Platelets, Red Cell Distribution Width (RDW) - by conductometric method after erythrocyte flotation.

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All the data were processed using statistical software Minitab 16.1 and analysed by ANOVA to establish statistically significant differences between groups.

RESULTS AND DISCUSSIONS

Growth performance results of pigs are shown in Table 2. It can be seen that all growth performance indicators were in favour of the experimental group. There were no statistically significant differences in the average final live weight, although it was higher by 6.52% in the group consuming probiotic. The average total gain and the average daily gain per pig between groups differ significantly (P = 0.031) by 11.17% and 11.14%. There was no difference in daily feed intake (n.s.). Average feed to gain ratio was better in the experimental group by 11.79%.

These results could be probably attributed to the use of the preparation Baykal EM-1. The Lactobacillus bacteria included in Baykal EM-1 increase the activity of bile, which promotes the absorption of fat-soluble vitamins A, E, D and fats. The intestinal micro flora is normalized, thus improving the nutrient absorption from feed.

Table 2. Weight development of weaned pigs, reared with and without the addition of Baykal EM-1 (mean ± SEM)

Indicators	Control group (n = 48)	Experimental group (n = 48)	Significance (P)
Average live weight at the beginning of the the experiment, kg	9.813±0.298	9.694±0.232	0.754
Average live weight at the end of the experiment, kg	24.131±0.878	25.813±0.572	0.112
Average total gain for the period, kg	14.319±0.724	16.119±0.391	0.031
Average daily gain, kg/pig/day	0.367±0.019	0.413±0.010	0.031
Average feed intake, kg/pig/day	0.959±0.024	0.952±0.023	0.830
Average feed/gain, kg	2.613	2.305	-

In a study conducted in Russia, it was found up to 70% increased instead of the usual 30% (SMR, 2005). At 4 months of age, the trial pigs, fed with Baykal EM-1, had a higher average live weight than the control group by 11% ($P<0.05$). These results are in one line with ours and with the results of a study of Dlamini et al. (2017). They observed no statistically significant differences in feed consumption between weaned pigs, fed with one or a combination of probiotics from the Lactobacillus group. However, there were statistically significant differences in average daily gain. Highest average daily gain - by 28.07% compared to the control group, by 29.82% compared to the group with *L. reuteri* and by 17.54% compared to the group with *S. salivarius* ($P<0.05$), had been obtained in the group consuming a combination of probiotics. The best feed utilization was registered in the same group. Improved growth performance of pigs was found in other studies (Huang et al., 2014; Zhao et al., 2018) and may be due to the effect of lactic acid bacteria included in the composition of the used probiotics. They are safe microorganisms with the ability to produce various inhibitory compounds, such as bacteriocins; organic acids such as lactic acid, hydrogen peroxide, diacetyl and carbon dioxide (Vieco-Saiz et al., 2019). They can inhibit harmful microorganisms with their arsenal or

through a mechanism based on competition for nutrients. Bacteria from the *Lactobacillus* group, through specific enzymatic functions (amylase, protease, etc.), can improve the absorption of nutrients, as well as stimulate the immune system of animals. It has been proven that the administration of *L. delbrueskii* subsp. *bulgaricus*, *L. acidophilus* and *L. casei* triggers the activity of macrophages in the body, enhances phagocytosis and increases cellular resistance of the organism (Chomakov et al., 1990). Our results from the study of the blood parameters of the animals, shown in Table 3, are in line to this statement. In both groups of pigs all values of blood parameters fit within the reference range (Friendship et al., 1984), with the exception of MCHC - the average concentration of hemoglobin in the erythrocyte, but it is calculable. They indicate that statistically significant differences, with a high degree of significance (P<0.001), were found in leukocyte content. It was higher by 19.83% in the trial group compared to the control group, and the lymphocyte content was higher by 20.91% (P<0.001). Similar results were found in the study of Lien (2012), examining the effect of probiotics and organic humic acids on blood parameters. The highest values of leukocytes in weaning pigs was measured in pigs consuming probiotics. Leukocytes' main function is to protect the organism from foreign invaders, such as bacteria, viruses and others. In this case, the higher content of leukocytes and in particular lymphocytes in the experimental group may be an indicator of better health, better adaptability and higher immunity than those of the control, due to the use of Baykal EM-1.

Table 3. Blood parameters in growing pigs, reared with and without the addition of Baykal EM-1 to the feed

Blood parameters	Control group		Experimental group		Reference values**
	Mean±SEM	C	Mean±SEM	C	
WBC/Leukocytes, G/L	20.25±0.82**a**	12.82	25.26±0.59**a**	7.32	8.7-37.9
LYM/Lymphocytes, G/L	10.48±0.35**a**	10.46	13.25±0.56**a**	13.29	2.2-16
MID/Monocytes, G/L	1.50±0.09	19.88	1.66±0.17	31.78	0.001-5.0
GRAN/ Granulocytes,G/L	8.28±0.29	10.83	8.52±0.13	4.97	
RBC/Red Blood Cells, T/L	6.63±0.32	15.28	6.56±0.15	7.22	5.3-8.0
HGB/Hemoglobin, G/L	119.30±5.89	15.60	114.40±3.67	10.14	90-140
HCT/Hematocrit, L/L	0.40±0.02	15.39	0.38±0.01	11.28	0.26-0.41
MCV/Mean corpuscular volume, fl	59.67±0.65	3.46	57.54±1.14	6.28	42-62
MCH/Mean corpuscular hemoglobin, pg	18.01±0.27	4.67	17.40±0.31	5.65	14-21
MCHC/Mean corpuscular hemoglobin concentration, g/L	301.80±2.17	2.28	302.70±1.37	1.44	320-360
RDW/Red blood cell distribution width, CV	0.16±0.01	9.25	0.17±0.01	20.94	
PLT/Platelet, G/L	564.10±54.48	30.54	618.90±91.99	47.00	
MPV/Mean platelet volume, fl	9.57±0.15	4.93	9.49±0.15	5.01	
PCT/Procalcitonin, L/L	0.23±0.01	16.01	0.23±0.01	18.81	
PDW/ Platelet distribution width, %	12.45±0.28	7.22	12.70±0.30	7.57	

Note: *Statistically significant differences are marked with the same letters, a - P<0.001
**Reference values are according to Friendship et al. (1984)

The results from this study indicate that the addition of Baykal EM-1, which is a mixture of probiotic strains and their products, in the feed for weaned pigs improved the immune system. They are in sync with those obtained by Dlamini et al. (2017), who found a higher content of immunoglobulin G in the blood of pigs consuming a combination of probiotics.

The results from growth performance and blood parameters indicate that a combination of probiotics added to weaned pig's diets has the potential to be used as a dietary supplement.

The results from the pig behavioural observations during the different periods of the experiment are shown in Figures 2 and 3.

The highest activity in both groups was observed in the hours of the feeding of the animals. In most of the cases it was in coincidence in the peak of movement.

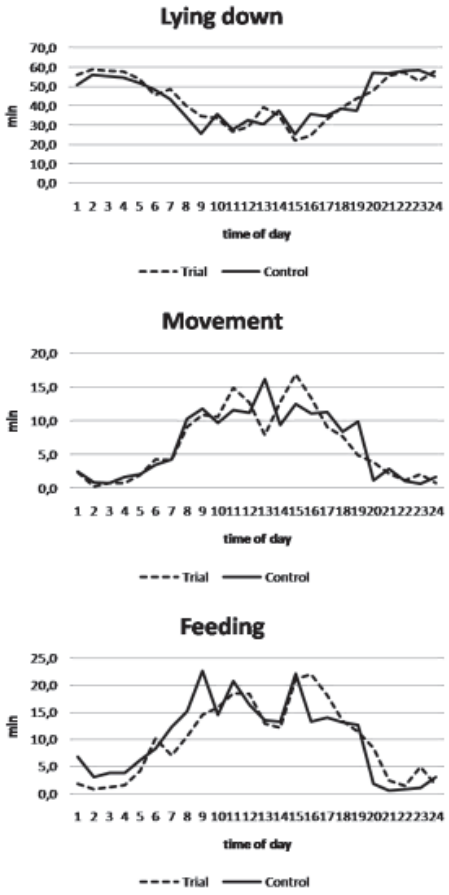

Figure 2. Behavioural reactions of growing pigs at the beginning of the experiment, reported at every hour (first observation)

The lowest values in the time of lying down was found in the time of feeding of the pigs. An interesting feature of the diagrams showing the movement of pigs in the two periods was that in the control group they are characterized by several peaks, and in the trial group the peak values were noted only at two points.

These details indicate that the animals in which the microbiological preparation was administered were calmer and moved only when their feed was being placed until all the animals in the pen were fed at the same time. In the control group, animals were active for longer periods of time - i.e. lower-ranking animals were able to eat "the leftovers", when leaders had already finished.

Although this evidence is circumstantial, it speaks to the better comfort of animals kept with the addition of a microbiological preparation in the feed, as it contributes to better absorption of nutrients.

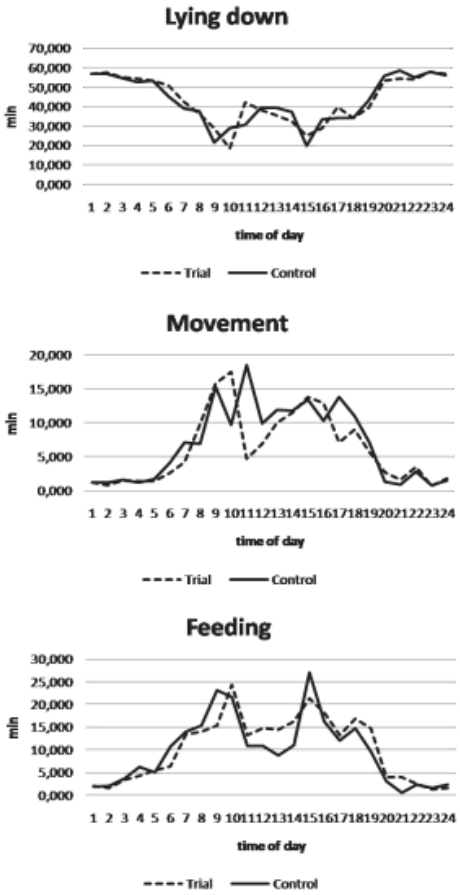

Figure 3. Behavioural reactions of growing pigs at the end of the experiment, reported at every hour (second observation)

CONCLUSIONS

The addition of Baykal EM-1 (10ml/kg feed) in weaned pig diets improved the average daily gain by 11.14% (P = 0.031).

The higher number of leukocytes and lymphocytes in pigs from the experimental group (P<0.001), compared to the control group, may be an indicator of better health and higher immunity.

A trend for better comfort in animals with the microbiological supplement in the feed, contributing to the better absorption of nutrients, has been established.

The results obtained in this study show that the combination of probiotics Baykal EM-1 has the potential to be used as a dietary supplement in weaned pigs.

ACKNOLEDGEMENTS

The authors acknowledge the kind support of a firm “Ecobalance Ltd.” and the provision of the probiotic preparation “Baykal EM-1” for the performance of the experiment.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

STUDY ON ANALYSIS OF BIOLOGICAL HAZARDS ASSOCIATED WITH COMPOUND FEED PRODUCING IN RELATION ON FOOD SAFETY

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Abstract

Compound feed and raw materials are potential vectors of pathogenic bacteria and potentially toxigenic fungi. The paper conducts a study on the production of compound feed in relation on food safety, by mycological and bacteriological analysis of samples of raw materials and compound feed for broilers, taken from a feed mill from Romania during 2019. Methodologically, the data were processed, analyzed and synthesized in the form of graphs and tables. The obtained results highlight during the analyzed period, in the samples of mycologically analyzed raw materials, eight genera of potentially toxigenic fungi were identified; the largest presence was the genus Aspergillus (64.45%). In the analyzed compound feed samples, four potentially toxigenic fungal genera were identified; in the samples with quantifiable results, the majority (61.9%) was identified the genus Aspergillus, and the lowest presence was the genus Cladosporium (9.53%). All results of bacteriological analysis aimed at determining the contamination with Salmonella spp. (54 analyzes for raw materials and 105 for compound feed) and E. coli (51 analyzes for raw materials and 101 for compound feeds) were negative. It can be concluded that in the production process of compound feed, the mycological, bacteriological and mycotoxicological analysis is must both for raw materials susceptible to contamination and of the finished products obtained; this goal is achieved in the unit studied, the results highlight the effectiveness of specific food safety control processes.

Key words*: feed safety, food safety, toxigenic fungi, Salmonella spp.*

INTRODUCTION

Feed safety is an important prerequisite for obtaining optimal production results as well as for maintaining the health of animals, especially in intensive industrial production, so it is necessary to constantly monitor raw materials and compound feed (Krnjaja et al., 2010). Compund feed and raw materials can be contaminated with undesirable substances, which may come from the environment or the production process (EFISC, 2014). Due to the relevant role of compound feed industry in the food chain, to ensure their safety, EC Regulation no. 183/2005 specifies, through Article 6 (1), that "Feed manufacturers shall establish, apply and maintain one or more permanent written procedures based on the HACCP principles."

Raw materials used to produce compound feed can come from various locations (Davies & Wales, 2013); if there has been exposure to wild or fecal animals, they may act as a source of non-endemic serotypes of *Salmonella* and other enteric bacteria, including the pathogenic *Escherichia coli* (Gosling et al., 2021). *Salmonella* can persist for many years in dry environments, such as those in feed mills, grain depots, and feed bunkers, and once it becomes resident, it can be difficult to eradicate (Davies and Wray, 1997). The presence of pathogens in compound feed may occur due to the use of contaminated raw materials during transport, in the production unit or on site. Because bacterial contaminants are unevenly distributed in the feed, the bacteria present may be damaged and difficulties may occur during microbial analysis. The purpose of controlling feed pathogens should be to ensure that feed contaminants are below a critical threshold to minimize the risk to human and animal health (Alali & Ricke, 2012).

Microbial contamination of feed is a potentially significant route for the entry of pathogens, including *Campylobacter* species, *Salmonella* enterica serotypes, *Escherichia coli* strains and *Yersinia enterocolitica*, into the human diet. Food-producing animals can be infected and

colonized with pathogens by ingesting contaminated feed; they can then be transmitted through the food chain to humans (Huss et al., 2015).

Contamination with potentially toxigenic fungi of feed is a regular occurrence worldwide and harmful effects have been observed in all classes of farm animals due to the production of mycotoxins by certain species and mold strains (D'Mello, 2004). Potentially toxigenic fungi are associated with cereals and oilseeds and mainly belong to the genera *Fusarium*, *Aspergillus* and *Penicillium* (Pacin, 2002). Factors that influence the development of microorganisms are represented by temperature, oxygen, relative humidity, water activity, pH, nutrients and different types of inhibitors (Savu & Georgescu, 2004).

Animals can become infected when they are fed compound feed contaminated with *Salmonella*; this can cause occasional clinical disease in some animals, but the major result is asymptomatic transmission. In addition, animals be infected by other animals infected with *Salmonella*, directly or through a contaminated environment for which the original source may have contaminated feed. *Salmonella* has been shown to be transmitted from feed to animals that consume it, and subsequently in food (EFSA, 2008). *E. coli* is a ubiquitous bacterium, present naturally in the human digestive tract in vast numbers, only a few strains being pathogenic and can induce symptoms; this serotype is of great importance for human and veterinary pathology, human becoming one of the most dangerous etiological agents of food poisoning (Savu & Georgescu, 2004).

Regulation (EC) no. Regulation (EC) No 2160 of 2003 on the control of *Salmonella* and other specific zoonotic agents present in the foodstuffs ensures that appropriate and effective measures are taken to detect and control them at all relevant stages of production, processing and distribution, including feed, to reduce their prevalence and the risk they pose to public health. In accordance with Article 5 (3) of Regulation (EC) No 183 of 2005 on feed hygiene, feed manufacturers must comply with specific microbiological criteria.

The paper conducts a study during 2019, on the production of compound feeds in relation to food safety, by mycological and bacteriological analysis of raw materials and compound feeds for broilers.

MATERIALS AND METHODS

Methodologically, the results of mycological and bacteriological analyzes performed throughout 2019 on raw materials and finished products from a compound feed factory in Romania were processed, synthesized and interpreted. Mycological analysis determined the content of raw materials and compound feeds in yeasts and molds, and bacteriological analysis determined the degree of contamination of raw materials and compound feed with *Salmonella* spp. and *E. coli*.

To obtain relevant results regarding the production of compound feed in relation on food safety, some raw materials susceptible to contamination (maize grain, wheat grain, soybean meal, sunflower meal) and the finished products obtained were analyzed, respectively, compound feed for broiler in different growth phases (starter, grower, finisher).

The analysis was performed in accredited specialized laboratories according to the following methods: SR ISO 21527–2: 2009 Microbiology of food and feed - Horizontal method for enumeration of yeasts and molds - Part 2: Technique for counting colonies in products with higher water activity less than or equal to 0.95; SR EN ISO 6579–1: 2017 Microbiology of the food chain. Horizontal method for the detection, counting and serotyping of S*almonella*. Part 1: Detection of *Salmonella* spp.; SR ISO 7251: 2009 Food microbiology; Horizontal method for the detection and enumeration of presumptive *Escherichia coli;* The least likely number technique.

The results obtained were compared with the values regulated by national and European legislation. The interpretation of the results led to the formulation of conclusions concerning the production of compound feeds in relation to food safety.

RESULTS AND DISCUSSIONS

The results of the mycological and bacteriological analysis performed for the samples of raw materials (corn grain, wheat

grain, soybean meal, sunflower meal) taken from the feed mill studied (Table 1), were presented for each month from January - December 2019.

Table 1. Results of biological analysis for raw materials

Specification		n[1]	MYCOLOGICAL ANALYSIS RESULTS		BACTERIOLOGICAL ANALYSIS RESULTS	
			Yeasts and molds (cfu/g) max. 5 x 10^3		*Salmonella* spp. max. absent/25 g	*Escherichia coli* cfu/g max. 10^2 cfu/g
			m[2] - M[3]	$\bar{x}$[4]		
Maize grain	Jan.	2	400	-	absent/25 g	-
	Feb.	3	4300	-	absent/25 g	0
	Mar.	6	700 - 1300	1000	absent/25 g	0
	Apr.	-	-	-	-	-
	May	5	900 - 3400	1966	absent/25 g	0
	June	-	-	-	-	-
	July	8	400 - 600	550	absent/25 g	0
	Aug.	-	-	-	-	-
	Sep.	4	800 - 1000	900	absent/25 g	0
	Oct.	1	800	-	-	-
	Nov.	2	800 - 3300	2050	-	-
	Dec.	1	400	-	-	-
Wheat grain	Jan.	2	400	-	absent/25 g	-
	Feb.	3	400	-	absent/25 g	0
	Mar.	6	600 - 2400	1500	absent/25 g	0
Soybean meal	Jan.	11	400 - 1000	625	absent/25 g	0
	Feb.	15	400 - 2900	980	absent/25 g	0
	Mar.	6	500 - 1000	750	absent/25 g	0
	Apr.	3	600	-	absent/25 g	0
	May	11	100 - 3400	1800	absent/25 g	0
	June	3	400	-	absent/25 g	0
	July	8	400 - 1636	1018	absent/25 g	0
	Aug.	13	400	400	absent/25 g	0
	Sep.	6	100 - 900	500	absent/25 g	0
	Oct.	3	500	-	absent/25 g	0
	Nov.	9	400 - 1550	850	absent/25 g	0
	Dec.	2	-	-	absent/25 g	0
Sunflower meal	Jan.	3	700	-	absent/25 g	0
	Feb.	6	400 - 800	600	absent/25 g	0
	Mar.	3	1600	-	absent/25 g	0
	Apr.	-	-	-	-	-
	May	2	-	-	absent/25 g	0
	June	3	1900	-	absent/25 g	0
	July	2	-	-	absent/25 g	0
	Aug.	-	-	-	-	-
	Sep.	3	400	-	absent/25 g	0
	Oct.	-	-	-	-	-
	Nov.	-	-	-	-	-
	Dec.	2	-	-	absent/25 g	0

[1]n = number of analysis
[2]m = minimum
[3]M = maximum
[4]$\bar{x}$ = average

The values for the quantitative determination of the contamination with yeasts and molds of the sampled corn grains varied between 400 cfu/g and 4300 cfu/g (n = 17), with an average of 1293 cfu/g, being below the maximum limit allowed by legislation (max. 5 x 10^3 cfu/g - the total amount of potential toxin-producing fungal species, according to Order MAFF 249/2003); for two analyzed samples (11.76%) the results were undetectable. All the results of the bacteriological analyzes (*Salmonella* spp. - n = 8 and *E. coli* - n = 7) performed for the maize grains registered negative values, being in accordance with the limits imposed by the legislation (absent/25 g *Salmonella* spp. and max. 10^2 cfu/g Escherichia coli according to Order MAFF 249/2003).

The results of mycological analyzes (n = 4) performed for wheat grains had values below the maximum limit imposed by the legislation, between 400 cfu/g and 2400 cfu/g, with an average of 950 cfu/g. All the results of the

bacteriological analyzes (*Salmonella* spp. - n = 4 and *E. coli* – n = 3) performed for the wheat grains registered negative values, being in accordance with the limits imposed by the legislation (absent/25 g *Salmonella* spp. and max. 10^2 ufc/g *E. coli*).

The resulting values for the concentration of yeasts and molds in soybean meal samples were below the limit imposed by the legislation in force (5 x 10^3 cfu/g), ranging between 400 cfu/g and 3400 cfu/g (n = 27), with an average of 865 ufc/g; for one analyzed sample (3.7%) the result was unquantifiable. All results of bacteriological analyzes to determine contamination with *Salmonella* spp. (n = 32) and *E. coli* (n = 31) were negative, in accordance with the maximum limits imposed by legislation (absent/25 g *Salmonella* spp. and max. 10^2 cfu/g *E. coli*).

The results for determining the content of yeasts and molds in sunflower meal had values below the maximum limit imposed by legislation, between 400 cfu/g and 1900 cfu/g (n = 7) with an average of 1040 cfu/g. All results of bacteriological analyzes to determine contamination with *Salmonella* spp. (n = 10) and *E. coli* (n = 10) were negative, in accordance with the maximum limits imposed by legislation.

According to the graphical representation of the number of potentially toxigenic fungal genera identified for each raw material analyzed (Figure 1), the content in the genus *Aspergillus* was predominant (65.45%) in all raw materials, followed by the content in the genus *Fusarium* (41.81%). The literature specifies that fungi of the genus *Aspergillus* are the main producers of aflatoxins (Marin et al., 2013), and ochratoxin A, the second most investigated mycotoxin after aflatoxins, in terms of its effect (Völkel et al., 2011).

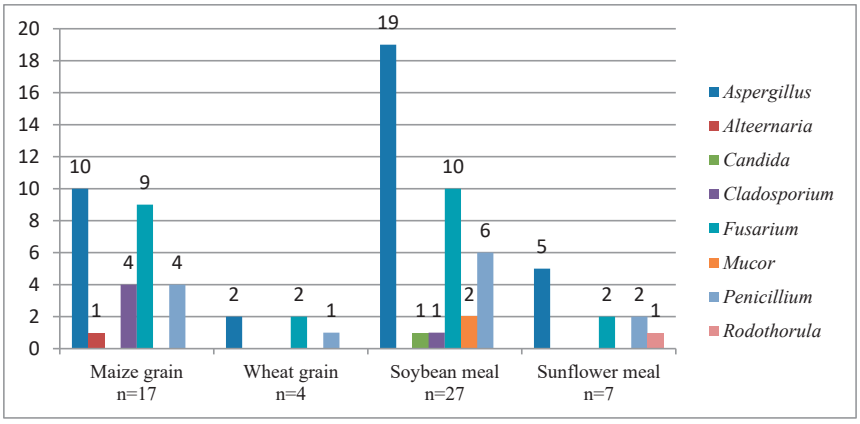

Figure 1. Number of fungal genera identified in raw materials

The results of mycological and bacteriological analyzes performed for the samples of compound feeds (starter, grower, finisher) from feed mill studied (Table 2), were presented for each month in which analyzes were performed, from January to December 2019.

The values found for the quantitative determination of the contamination of starter compound feed with yeasts and molds, varied between 100 cfu/g and 2900 cfu/g (n = 30), resulting in an average of 507.6 cfu/g, in accordance with the maximum regulated limits (max 5 x 10^3 cfu/g - the total number of potentially toxin-producing fungal species according to Order MAFF 249/2003); for 16 samples analyzed (53.3% of the total) the results were undetectable.

Table 2. Results of biological analysis of compound feed

Specification		n[1]	MYCOLOGICAL ANALYSIS RESULTS		BACTERIOLOGICAL ANALYSIS RESULTS	
			Yeasts and molds (cfu/g) max. 5 x 10^3		*Salmonella* spp. max. absent/25 g	*Escherichia coli* cfu/g max. 10^2 cfu/g
			m[2] - M[3]	$\bar{x}$[4]		
Starter compound feed	Jan.	8	100 - 2900	1133	absent/25 g	0
	Feb.	6	400	400	absent/25 g	0
	Mar.	3	nd[5]	-	absent/25 g	0
	Apr.	12	100 - 400	325	absent/25 g	0
	May	15	100 - 400	280	absent/25 g	0
	June	3	800	-	absent/25 g	0
	July	6	nd	-	absent/25 g	0
	Aug.	10	400	-	absent/25 g	0
	Sep.	9	nd	-	absent/25 g	0
	Oct.	6	400	400	absent/25 g	0
	Nov.	9	nd	-	absent/25 g	0
	Dec.	3	400	-	absent/25 g	0
Grower compound feed	Jan.	5	nd	-	absent/25 g	-
	Feb.	9	400	400	absent/25 g	0
	Mar.	9	400 - 600	500	absent/25 g	0
	Apr.	3	400	-	absent/25 g	0
	May	9	400	-	absent/25 g	0
	June	3	nd	-	absent/25 g	0
	July	9	400	400	absent/25 g	0
	Aug.	3	nd	-	absent/25 g	0
	Sep.	6	nd	-	absent/25 g	0
	Oct.	-	-	-	-	-
	Nov.	12	400	400	absent/25 g	0
	Dec.	9	nd	-	absent/25 g	0
Finisher compound feed	Jan.	8	400	-	absent/25 g	0
	Feb.	21	400	400	absent/25 g	0
	Mar.	24	400	-	absent/25 g	0
	Apr.	6	400	-	absent/25 g	0
	May	12	400	-	absent/25 g	0
	June	9	nd	-	absent/25 g	0
	July	18	400	-	absent/25 g	0
	Aug.	9	nd	-	absent/25 g	0
	Sep.	24	400 - 700	475	absent/25 g	0
	Oct.	-	-	-	-	-
	Nov.	6	400 - 500	450	absent/25 g	0
	Dec.	6	500	-	absent/25 g	0

[1]n = number of analysis; [2]m = minimum; [3]M = maximum; [4]$\bar{x}$ = average; [5]nd = not detectable

All results of bacteriological analyzes to determine *Salmonella* spp. (n = 31) and *E. coli* (n = 29) for starter compound feeds had negative values, in accordance with the limits allowed by law (absent/25 g *Salmonella* spp. and max. 10^2 cfu/g *E. coli* according to Order MAFF 249/2003).

The results for determining yeasts and molds in grower compound feed, had values between 400 cfu/g and 600 cfu/g (n = 26) with an average of 416 cfu/g. All results of bacteriological analyzes to determine contamination with *Salmonella* spp. (n = 26) and *E. coli* (n = 25) were negative. The values for the quantitative determination of finisher compound feed with yeasts and molds, varied between 100 cfu/g and 700 cfu/g (n = 40), with an average of 380 cfu/g; for 30 samples analyzed (62.5% of the total) the results were undetectable. All results of bacteriological analyzes to determine the contamination with *Salmonella* spp. (n = 48) and *E. coli* (n = 47) performed for compound feeds recorded negative values.

The graphical representation of the number of potentially toxigenic fungal genera identified (Figure 2) for each type of compound feed (starter, grower, finisher) analyzed, reveals that the genus *Aspergillus* was most often identified (61.9%) in the samples that had results quantifiable, followed by the genus *Penicillium* (33.3%).

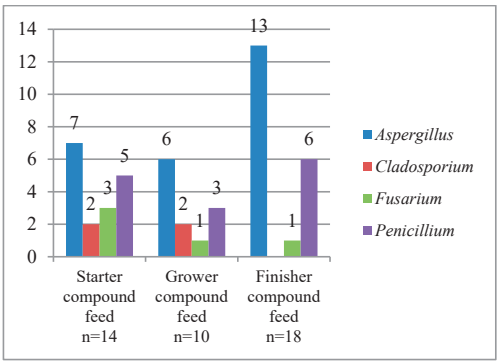

Figure 2. Number of fungal genera identified in compound feed

CONCLUSIONS

Compound feed and raw materials represent a favorable environment for the development of potentially toxigenic fungi and bacteriological contaminants, and therefore permanent analysis is needed to control them.

In the samples of raw materials analyzed, eight genera of potentially toxigenic fungi were identified: *Aspergillus*, *Alternaria*, *Candida, Cladosporium*, *Fusarium*, *Mucor*, *Penicillium* and *Rodothorula*; the highest presence was the genus *Aspergillus* (64.45%) followed by the genus *Fusarium* (41.81%), and the lowest presence was the genus *Rodothorula* (1.81%).

In the samples of compound feed analyzed, four genera of potentially toxigenic fungi were identified: *Aspergillus*, *Cladosporium*, *Fusarium* and *Penicillium*; the most present was the genus *Aspergillus* (61.9%), and the lowest presence was the genus *Cladosporium* (9.53%).

Regarding the correlation of the results of mycological analyzes with the maximum limits allowed by the legislation, it was found that they were within the regulated values, both for raw materials and for compound feeds.

Regarding the results of bacteriological analyzes aimed at determining the contamination with *Salmonella* spp. and *E. coli*, for raw materials and compound feed studied no positive values were registered.

Although no results were found above limits allowed by the legislation, the frequency of identification of mycotoxin-producing fungal genera is not negligible.

It can be concluded that in the production process of compound feeds, the mycological, bacteriological and mycotoxicological analysis is must both for raw materials susceptible to contamination and of the finished products obtained, in order to ensure public health; this goal is achieved in the unit studied, the results highlighting the effectiveness of specific food safety control processes.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

CHARACTERIZATION OF PHYTOCHEMICALS PRESENT IN WINERY BY-PRODUCTS WITH NUTRITIONAL POTENTIAL FOR ITS USE AFTER THE AGRO-INDUSTRIAL PROCESS

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Abstract

The winery by-products contain phytochemicals that are characterized as bioactive components that exhibit nutritional properties. The natural bioactive compounds and nutritional properties of winery's by-products vary with the grape variety and wine making method. The aim of this work was to characterize the nutritional value of grape marc (GM) and yeast biomass (YB) from white wine making using Feteasca Regala variety. Polyunsaturated fatty acids around 67.65% of lipids in GM are essential fatty acids representing phytochemicals with antioxidant properties (omega 6 representing 66.95% of lipids) compared to YB (29.88% and omega 6 representing 24.33% of lipids). YB have a high protein percentage (16.58%) compared to grape marc (7.85%) with high concentrations of essential amino acids, being glutamic acid, aspartic acid, lysine and leucine the most abundant. Infrared FTIR-ATR spectroscopy has been used to determine the functional groups in GM and YB, which show, in addition to the presence of polyphenols, the characteristic compounds that are part of soluble dietary fiber and α and β-glycosidic picks of polysaccharide contents. Due to its phytochemical content, the residues from the wine industry provide essential nutrients for metabolism and can be considered valuable raw materials for their use in animal consumption products.

Key words*: grape mark, yeast biomass, phytochemicals, winery's by-products.*

INTRODUCTION

The circular economy needs a lot of effort and involvement among companies to create a reliable and usable recovery process for waste and by-products. The management of waste or by-products can create environmental problems that demand revaluation methodologies in order to become sources of wealth thanks to the revalued product (Fernández et al., 2008). Wine cellar by-products such as skins, seeds, and yeast biomass (YB) are important source of phytochemical compounds with high functional value such as proteins, essential fatty acids, fibre, and minerals. In this sense, these by-products could be used as valuable raw materials for human or animal nutrition (Marinescu et al., 2019; Milner, 2004). Feteasca Regala variety is one of the most cultivated grape varieties in Romania. With a relatively high production rate, it is a very common and highly appreciated variety with a massive production of wine which results in large amounts of by-products. In general, the yield of grape marc (GM) production (by-product obtained after vinification) is about 25-30 kg per 100 kg of grape. From this residue, half are grape skins, 25% stems, and 25% seed (Maicas & Mateo, 2020).

Oenological industry mainly generates solid waste (GM) and liquids (YB) that have a high organic pollutant load and a high concentration of solids. In this case, the legislation is very demanding since it requires a special pre-treatment of this waste. In the case of GM, this is often used to obtain alcohol, and the rest left after this process is usually used as a nutritional supplement in livestock feed. The seeds can be

used to obtain oil and extracts, and the stem can be used in the diet of animals or as biomass. In spite of these advantages, the proteins in grape seeds are considered indigestible, and this is attributed to the strong interaction between proteins and tannins, which limits the digestibility of proteins since tannins are thought to be inhibitors of digestive enzymes (Yu & Ahmedna, 2013). It is known that compared to cereals, the dietary fiber of fruits and vegetables has a higher nutritional quality (Yi et al., 2009). The GM has a significant percentage of dietary fiber, which includes hemicellulose, cellulose, pectin and lignin. Dietary fiber depends on the variety of grapes, white GM having lower fiber concentration (17-28%) than red GM (50-56%), and most of it (95%) is insoluble fiber. Taking into account the large amount of GM that is generated worldwide each year, these have great potential as an important source of insoluble fiber for the development of functional foods (Saura-Calixto, 2011; Yu & Ahmedna, 2013). Grape seeds also contain 7% complex phenols, 16% oil, 11% protein, sugars and minerals. 63% of the total phenols of the red varieties vines are found in the seeds, 34% in the skins and 3% in the must (Murga et al., 2000).

Yeast biomass residue (YB) has a high percentage of alcohol (7%), dietary fiber (20% hemicellulose, 8% cellulose and 13.5% lignin), tartaric acid (20-30%) and proteins (15%). Gomez et al., (2004) shows that YB has a 5.4% lipid content, where palmitic (33%) and linoleic (21%) acids seem to be the most abundant.

FTIR spectroscopy in conjunction with chemometrics represents a valuable tool for monitoring the composition of wine by-products. Grape seeds of grape marc were also studied by FTIR spectroscopy and bioactive compounds were identified as gallic acid carboxylate and proanthocyanidin gallate groups, fatty acid methyl esters, extractable polyphenols in red grape skins as well as other attributes in the grape skin and grape seed (Nogales-Bueno et al., 2017).The ATR-FTIR techniques have been widely used for the analysis of microorganisms to determine their cellular components, such as the cell membrane and wall, thus allowing their classification (Oust et al., 2004). It has also been used to study the autolysis of *Saccharomyces cerevisiae* in sparkling wines and the biochemical changes in different stages of the alcoholic fermentation process (Burattini et al., 2008; Cavagna et al., 2010).

The winery by-products (GM and YB) can be used in animal feed, for improvement of the capacity against the oxidation of lipids and proteins, as well as for coloring the meat, and also in feeding of the laying hens, improving the size of the egg. In addition, the dietary fiber, which is a group formed by free polysaccharides resistant to enzymatic hydrolysis at the gastrointestinal level, are introduced in the animal diet (DeVries, 2004).

The objective of this study was to determine the content of phytochemicals in the residual biomass of yeast (YB) and grape marc (GM) resulted from the vinification process of the Feteasca Regala variety obtained from the Controlled Designation of Origin of Wines in Pietroasa vineyard, Romania in order to evaluate the nutritional value of these two by-products in animal feed.

MATERIALS AND METHODS

The samples of residual biomass of yeast (YB) and grape marc (GM) resulting from the winemaking process of the white grape variety Feteasca regala (FR) were obtained in 2018 from Controlled Designation of Origin of Wines in Pietroasa vineyard, Romania.

Determination of phytochemical content in YB and GM winery by-products of FR grapes variety

The YB and GM samples taken in the study were distributed in sterile, single-use Petri dishes and placed in the dehydrator (Gorenje FDK24DW food dehydrator) at a temperature of 55°C for 24 h (MARIN et al., 2019). The dried YB and GM samples were analyzed for the content of moisture, crude ash, crude protein, crude fat, total sugars, and total amino acids, according to the requirements specified by the Commission Regulation (EC) 152/2009.

Determination of moisture and ash

The moisture content was determined through drying of the samples at 103^0C for four hours, according to Commission Regulation (EC) 152/2009.

The determination of the crude ash (%) was carried out by the gravimetric method, according to Regulation (EC) 152/2009 and the standard SR EN ISO 2171: 2010. It was performed through the ashes of the samples at 550^0C.

Determination of crude protein content

The determination of the crude protein content was performed according to the Kjeldahl method, in accordance with the standard SR EN ISO 5983-2: 20101 and the Regulation (EC) 152/2009. The crude protein is obtained based on the nitrogen content determined by the Kjeldahl method multiplied by the factor 6.25.

Determination of total sugar content

The total sugars, expressed as glucose, were determined using the Luff-Schoorl method, in accordance with Commission Regulation (EC) 152/2009.

Determination of crude fat content

The crude fat content was determined based on the Procedure B described in the Regulation (EC) 152/2009, using the extraction with prior hydrolysis.

Determination of NDF and ADF content

The fibrous fractions (g/100g)- the neutral detergent fibre content (NDF) and the acid detergent fibre content (ADF) - were determined by gravimetric determination, using the AOAC Official Method 2002-04 and the AOAC Official Method 973.18, respectively.

Determination of fatty acids and amino acid content

The fatty acids spectrum was determined by GC, using a 456-SCION gas chromatograph configured with FID. The principle of the method consists in the transformation of glycerides from fats and oils into methyl esters by dissolution in organic solvent and transesterification with methanolic potassium hydroxide, followed by the separation of methyl esters from the sample by gas chromatography on capillary chromatographic column with very polar stationary phase. The method is applied to determine the content of fatty acids, including the saturated, monounsaturated and polyunsaturated fatty acids, as well as the omega 3, omega 6, omega 9 fatty acids and *Trans* fatty acids. In addition, the amount of saturated, monounsaturated, polyunsaturated, *Trans* fat, as well as omega 3, omega 6 and omega 9 fat can be also calculated by reporting the fatty acid content to the extracted fat.

The total amino acids content was determined in accordance with Regulation (EC) No. 152 (2009), after hydrolysis. The working method complies with the standards SR CEN ISO/TS 17764-1:2008 - Animal feeding stuffs - Determination of the content of fatty acids - Part 1: Preparation of methyl esters, and SR CEN ISO/TS 17764-2:2008 - Animal feeding stuffs - Determination of the content of fatty acids - Part 2: Gas chromatographic method.

The content of fatty acids, saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids, and *Trans* fatty acids is calculated as the sum of the relative mass fractions of the corresponding methyl esters according to the ratio obtained after gas-chromatographic analysis. The result is reported with an accuracy of 0.01% mass fraction.

The amount of saturated fats, monounsaturated fats, polyunsaturated fats, *Trans* fats, omega 3 fats, omega 6 fats and omega 9 fats are determined by reporting the fatty acid content to the fat content of the sample and is expressed in g/100 g product with an accuracy of 0.01 g/100 g.

Spectroscopic analysis for YB and GM winemaking residue powdered samples from FR grapes variety

Infrared spectroscopy (ATR-FTIR) was used to qualitatively determine the chemical functional groups of two winemaking by-products powdered samples, yeast biomass residue (YB) and grape marc residue (GM) from white Feteasca Regala (FR) grapes variety. No other sample preparation was performed for spectral analysis. The spectra of each sample were collected with a Thermo Scientific (Waltham, MA, USA) Nicolet iS50 FT-IR spectrometer equipped with an inbuilt diamond attenuated total reflection (ATR) system with a refractive infrared beam bouncing off a 45 angle of incidence. The instrument was fitted with OMNIC software (OMNIC 7.3, Thermo Fisher Scientific Inc.). Before measuring each sample of vinification residues, the background was carried out. The spectral range for each sample was between 400-4000 cm^{-1} with an average of 64 scans and a resolution of 8 cm^{-1} at a scanner velocity of 7.5 kHz.

Statistical analysis

Each sample analysis was worked out in triplicate. The data reported are means (± SD) of at least three independent experiments.

RESULTS AND DISCUSSIONS

Although the phytochemicals content is higher in grapes, in GM and YB by-products generated in winemaking they are also present, since part of them are transmitted to the wine during the vinification process (Alonso et al., 2002; Spanghero et al., 2009). The content depends on the grape variety and is influenced by location, climate, and state of maturity of the grapes and the fermentation process, as well as by the processing and production of wine (red or white grapes, extraction, drying etc.) (Ojeda et al., 2002; Muñoz et al., 2004; Baumgärtel et al., 2007).

Phytochemical content in YB and GM winery by-products of white grapes FR variety

Table 1 presents the phytochemical compositions of YB and GM of the white grape FR variety. Both powder residues GM and YB have a dry matter content of approx. 95%, and the ash content was higher in YB residue (14.92%) than in GM residue (4.15%).

Table 1. Biochemical compositions of winery by-products (YB and GM) from FR grapes variety

Parameters	YB	GM
Ash (%)	**14.92**	4.15
Moisture (%)	3.87	**7.49**
CP (%)	**16.58**	7.85
TS (g/100 g)	0.71	**20.00**
Lipids (g/100 g)	3.30	**5.23**
NDF (g/100 g)	16.15	**41.01**
ADF (g/100 g)	4.80	**32.55**

Values are expressed as mean; FR: Feteasca Regala variety; YB: yeast biomass; GM: grape marc; CP: crude protein; TS: total sugar; NDF: Neutral Detergent Fiber; ADF: Acid Detergent Fiber.

The CP content in YB was twice higher (16.58%) than in the GM residue (7.85%), but all the other parameters analysed presented higher values in the GM residue than in the YB residue, highlighting TS content (20 g/100 g) and dietary fiber values (71.74% NDF, respectively 87.14% ADF for the total). The winery by-products can be used as feed, knowing that there are differences between the nature of dietary fiber, cereals being the main sources of cellulose and hemicellulose, fruits and vegetables are mainly pectin (Elleuch et al., 2011). The cell wall specific carbohydrates (polysaccharide) (e.g. β-glucans) have not been determined and therefore these are not included in the composition presented in table 1.

Other authors highlight that GM contains 40% fermentable carbohydrates (by dry weight) from white grapes and 4.6% from red grapes (Llobera et al., 2007). Dietary fiber has different components with physical, chemical and physiological properties, such as oligosaccharides, lignin, resistant starch and associated plant substances and depends on the grape variety (17.3-28% white grape and 51.1-56.3% red grape) (Deng et al., 2011; Bravo and Saura-Calixto, 1998).

Fatty acids and their profile in YB and GM winery by-products of white grapes FR variety

Both content and composition of fatty acids in GM and YB depend on the variety and maturity of the grape (Yu and Ahmedna, 2013; Bravi et al., 2007). GM highlighted for its PUFA content (67.65% lipids), omega 6 acids represented 98.96% of PUFAs, compared to YB (PUFA 29.88% of lipids of which 81.42% were omega 6 acids). In the case of omega 3 acids, the values were higher (5.55% of lipids) in YB residue and in GM residue were almost non-existent or at very low values (0.70% of lipids) (Table 2).

Table 2. Total fatty acid contents (% of lipids) in winery by-products (YB and GM) from FR grapes variety

Fatty acids (% of lipids)	YB	GM
Σ SFA	30.14	12.88
Σ MUFA	23.26	19.38
Σ PUFA	**29.88**	**67.65**
Σ FAT	4.18	0.82
Σ acid C18:2 trans	3.23	nd
Σ acid C18:3 trans	nd	0.09
Σ Ω 3	**5.55**	0.70
Σ Ω 6	24.33	**66.95**
Σ Ω 9	14.95	**18.17**

Values are expressed as mean; FR: Feteasca Regala variety; YB: yeast biomass; GM: grape marc; SFA: Saturated fatty acid; MUFA: Monounsaturated fatty acid; PUFA: Polyunsaturated fatty acid; FAT: Fatty acid Trans; nd: Not detected.

The profile of the content of fatty acids in the residues of GM and YB of white grapes of the FR variety is presented in Table 3.

Table 3. Profile of the fatty acid contents (% of lipids) in winery by-products (YB and GM) from FR grapes variety

Fatty acids (% of lipids)	YB	GM
Caprinic acid (C10:0)	0.58	nd
Lauric acid (C12:0)	0.94	nd
Myristic acid (C14:0)	0.87	0.18
Myristoleic acid (C14:1)	0.25	nd
Pentadecanoic acid (C15:0)	0.06	nd
Palmitic acid (C16:0)	**16.61**	**8.32**
Palmitoleic acid (C16:1)	6.85	0.39
Margaric acid (C17:0)	0.13	nd
Cis Heptadecenoic acid (C17:1)	0.14	nd
Stearic acid (C18:0)	**6.74**	**4.00**
Oleic acid (C18:1 cis)	**14.78**	**17.69**
Vaccenic acid (C18:1 trans)	0.91	0.82
Linoleic acid (C18:2 cis)	**19.84**	**66.95**
α- Linolenic acid (C18:3n-3)	**4.59**	0.70
γ-Linolenic acid (C18:3n-6)	**4.33**	nd
Arachidic acid (C20:0)	1.59	0.26
Eicosenoic acid (C20:1 n-9)	0.13	0.15
Eicosadienoic acid (C20:2 n-6)	0.06	nd
Eicosatrienoic acid (C20:3 n-3)	0.14	nd
Arachidonic acid (C20:4n-6)	0.10	nd
Eicosapentaenoic acid (C20:5n-3)	0.58	nd
Heneicosanoic acid (C21:0)	0.33	nd
Docosanoic (Behenic)acid (C22:0)	**1.48**	0.12
Docosapentaenoic acid (C22:5 n3)	0.24	nd
Tricosanoic acid (C23:0)	0.24	nd
Lignoceric acid (C24:0)	0.53	nd
Nervonic acid (C24:1)	Nd	0.33
Caprilyc acid C8:0 -	0.04	nd

Values are expressed as mean; FR: Feteasca Regala variety; YB: yeast biomass; GM: grape marc; nd: Not detected.

The results show that in GM, due to the high content of linoleic acid (66.95% of lipids), there is a high content of omega 6 fatty acids, due to the presence of the seeds (Marin et al., 2019). The main essential fatty acids found are linoleic (66.95% of lipids), oleic (17.69% of lipids), palmitic (8.32% of lipids) and stearic (4.00% of lipids). The YB residue contains fatty acids in a lower quantity but a wider variety and the α-linolenic acid and γ-linolenic acid contents are higher (4.59%, respectively 4.33% of lipids) than in GM (0.70% of lipids, respectively not detected), belonging to acids omega 3. It is remarkable that unsaturated fatty acids predominate, representing an important source of essential fatty acids for the feed industry (Marin et al., 2019; Barbulescu et al., 2020; Teodorescu et al., 2020).

The incorporation of grape marc in the diet of ruminants, as shown by Jerónimo et al. (2012), can interfere with the lipid profile of meat due o the presence of active phytochemical components. The author observed an increase in unsaturated fatty acids (long chain n6) when including 25 g kg^{-1} dry matter of grape seed extract in lamb diets. Another study demonstrates a lower level of oxidation in turkey meat hamburger by ingestion or post-mortem addition of grape seed extract, and also blue fish supplemented with grape seed extract (100 ppm) keeps its freshness for 10 days of conservation at 4ºC, in comparison with the control (3 days) (Cagdas & Kumcuoglu, 2015).

Amino acids content in YB and GM winery by-products of white grapes FR variety

Rao (1994) and Zhou et al. (2010) showed that the concentration and composition of amino acids vary with the variety of the grape, growing area, fertilization etc. Our previous studies showed slightly higher values of total essential amino acids in GM residues of the red varieties compared to white varieties (1 g/100 g difference) (Marin et al., 2019). In the case of the FR variety and for the two residues analyzed, higher values were observed in YB residues compared to GM residues (table 4). Essential amino acids, important in nutrition (Castro Sousa et al., 2014), the most abundant founded were aspartic acid (1.54 g/100 g, respectively 8.87 g/100 g), glutamic acid (1.35 g/100 g, respectively 1.08 g/100 g), serine, alanine and leucine.

Table 4. Essential amino acids content (g/100g) in winery by-products (YB and GM) from FR grapes variety

Essential amino acids (g/100 g)	YB	GM
Aspartic acid	**1.54 ±0.19**	0.87 ±0.11
Threonine	0.85 ±0.10	0.43 ±0.05
Serine	**1.33 ±0.16**	0.73 ±0.09
Glutamic acid	**1.35 ±0.17**	**1.08 ±0.13**
Proline	0.60 ±0.07	0.76 ±0.09
Glicine	0.83 ±0.10	0.65 ±0.08
Alanine	**0.94 ±0.12**	0.46 ±0.06
Valine	0.70 ±0.09	0.24 ±0.03
Isoleucine	0.66 ±0.08	0.46 ±0.06
Leucine	**1.10 ±0.13**	**0.92 ±0.11**
Tyrosine	0.60 ±0.07	0.11 ±0.02
Phenylalanine	0.60 ±0.07	0.30 ±0.04
Lysine	**1.10 ±0.13**	0.41 ±0.05
Histidine	0.35 ±0.04	0.19 ±0.02
Arginine	0.62 ±0.07	0.46 ±0.06

Data are expressed as mean ± SD; FR: Feteasca Regala variety; YB: yeast biomass; GM: grape marc.

Spectroscopic analysis of YB and GM winery by-products of white grapes FR variety

FTIR spectroscopy is a suitable tool for applications in the oenological sector (Lucarini et al., 2020). Fourier transform infrared spectroscopy (ATR-FTIR) was used for qualitative analysis of the YB and GM residue samples obtained in the vinification process of the white grape FR variety (Figures 1 and 2).

The differences between the samples of the two residues (YB and GM) were determined with the help of the peaks found in the spectral region of 4000-500 cm^{-1} (Basalekou et al., 2015). Table 5 collect the detailed peak positions and band assignments to compare the two residues.

Our study compared the specific adsorption profiles identified between the two residues analyzed in which the group's functional characteristics absorb for each of the residues. In general, the spectres seem to have the peaks in the same areas with respect to the positions of the spectral bands, thus we can identify the signatures of the main functional groups for the two residues. Differences can be seen between the shapes of the bands and the relative intensities in the spectra.

In the case of YB residue, irradiation with infrared light at different wavelengths exhibited well-defined spectral regions corresponding to the vibration determined by the components of the cells (proteins, fatty acids, polysaccharides, chitins, glucans among others) Grangeteau et al. (2015 and 2016). Characteristic vibrations were observed with very sharp peaks due to proteins in the spectral region 1621 cm^{-1} (amide I) and 1540 cm^{-1} (amide II), 1131 cm^{-1} (nucleic acids) and for carbohydrates the bands between 903-1299 cm^{-1} representing the content of α and β-glucans. β-glucans of a typical yeast cell consist of ~30-45% of β-1, 3 glucan and ~5-10% of β-1, 6 glucan (Soares and Soares, 2012). β-glucans, the main components of yeast biomass, enhance the innate immune response, since mammalian cells lack this component (Chan et al., 2009).

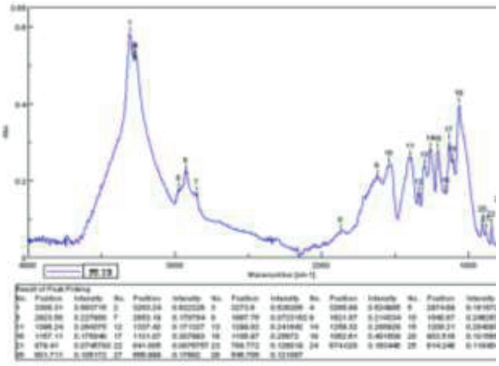

Figure 1. ATR-FTIR spectral analysis of the winemaking by-products (YB) from FR grapes variety

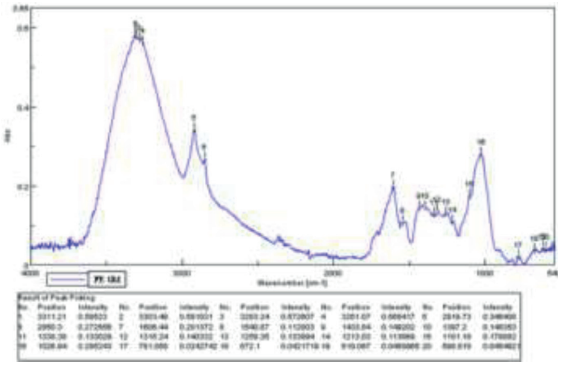

Figure 2. ATR-FTIR spectral analysis of the winemaking by-products (GM) from FR grapes variety

Table 5. Wavenumber (cm^{-1}) assignments of ATR-FTIR spectra of YB and GM powders from FR grapes variety with different sizes

Wavenumber (cm^{-1})				Assignments
YB		GM		
Positions	Intensity	Positions	Intensity	
3308	**0.5837**	3311	0.5852	
3283	0.5222	3303	**0.5810**	–OH stretch vibration, -H bonded νOH=3200-3600 cm^{-1}
3273	**0.5262**	3283	0.5226	
3265	0.5249	3261	**0.5654**	
2976	0.1815			
2926	0.2279	2919	**0.3464**	C–H stretching, aliphatic
2853	0.1707			
		2350	**0.2725**	
1621	**0.2143**			Aromatic CH bonds (COO^-)
		1606	**0.2013**	
1540	**0.2462**	1540	0.1120	C = C stretching bands
		1433	**0.1492**	Antisymmetric in-plane bending of $–CH_3$
1396	**0.2643**	1397	0.1483	Symmetric in-plane bending of $–CH_3$
1337	**0.1713**	1315	0.1403	
1299	**0.2418**			
1259	**0.2859**	1258	0.1338	Stretching vibration of C–O
1208	**0.2840**	1213	0.1136	
1131	**0.3079**			
1106	**0.2567**	1101	0.1788	
1062	**0.4015**			Stretching vibration of C–O
		1026	**0.2852**	
903	**0.1015**			
788	**0.1260**	781	0.0242	CH out of-plane conformations
674	**0.1184**	672	0.0421	
601	**0.1051**			
566	**0.1769**			
545	**0.1210**			

Also peaks with high absorption intensity were found in the spectral region 3308-3265 cm^{-1} (NH of proteins), 2976-2853 cm^{-1} (CH of lipids and proteins).

GM residue analyzed by ATR-FTIR showed the presence of absorption peaks at 3311-3261, 2919, 2350, 1606, 1540, 1433, 1397, 1258, 1101, 1026, 781 and 672 cm^{-1}. The bands with the most absorbing peaks are found at 3311, 3303, 3283 and 3261 cm^{-1} corresponding to the -OH stretching vibration of the phenolic structures. At the 2919 cm^{-1} absorption peak, this is due to symmetric and asymmetric C-H stretching bonds in the CH_2 and CH_3 groups, methyl band bonds belonging to lipids (Ricci et al., 2015). The 1433-781 cm^{-1} region represents the *fingerprint* that provides important information on the organic compounds (sugars, alcohols, and organic acids) present in the sample. The food industry and farming should interact each other to benefit both from this interaction, since both have problems disposing of their waste. By-products of the winery, it is possible to use them as feed with antioxidant functions, improving their behaviour against the oxidation of lipids and proteins, and the colour of the meat (Jerónimo et al., 2012).

CONCLUSIONS

Our study shows a high content of polyunsaturated fatty acids (PUFA) in the main

residues of the winery, GM (67.65% of lipids) and YB (29.88% of lipids). Among these the most important and with the highest content is linoleic and oleic acid (66.95%, respectively 17.69% of lipids) in GM, which are precursors for omega 6 fatty acids, due to the presence of the seeds.. Precursors for fatty acids omega 3 were observed in YB as well, α-linolenic acid and γ-linolenic acid (4.59%, respectively 4.33% lipids). The results showed a higher dietary fiber GM content (41.01 g/100 g NDF and 32.55 g/100 g ADF) compared to YB content (16.15 g/100 g NDF and 4.80 g/100 g ADF). Due to the content of dietary fiber in grape marc, with a high content of extractable and non-extractable phenolics, this is important in lipid metabolism.

ATR-FTIR provides molecular vibrations (stretching, bending and torsion) of the phytochemicals present in the by-products of the winery (GM and YB) representing the molecular *fingerprint* of the sample. The YB residue spectra are well defined with very marked peaks, representing cell components: 1621 cm^{-1} (amide I); 1540 cm^{-1} (amide II); 1131 cm^{-1} (nucleic acids) and 903-1299 cm^{-1} (carbohydrates; α and β-glucans). Peaks very sharp bands between 3308-3265 cm^{-1} represent stretching NH of proteins and CH of lipids and proteins. The peaks of the GM residue recognize the -OH stretching of phenolic structures, polysaccharides and/or lignin (3311-3261 cm^{-1}) This residue contains grape seeds with beneficial PUFA fatty acid content, demonstrated by stretching vibrations (2919 cm^{-1}) symmetric and asymmetric CH (CH_2 and CH_3). Study of the content in phytochemicals of YB and GM demonstrated the antioxidant properties of these residues, in addition to providing essential nutrients for the proper functioning of the metabolism with a healthy effect in the medium and long term.

ACKNOWLEDGEMENTS

This work was supported by the project 20PFE/2018 "Development of the Center for the superior valorisation of the by-products from the vineyard farms", Program 1 -Development of the National Development Research System, Subprogram 1.2 -Institutional Performance - Institutional Development Projects - Funding Projects for Excellence in Institutional Development Research, PNCDI III.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

CHARACTERISTICS OF THE COMPOSTION AND BIOACTIVE PROPERTIES OF MOUNTAIN MILK USED FOR EMMENTAL CHEESE MAKING - REVIEW

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Abstract

Wild mountain pastures are situated at high altitudes (800-1500 m), where they benefit from a rich and diversified flora, with terpenes and propionic bacteria that are found in salty soils, poor in iron and manganese, these conditions being crucial for the process of obtaining the Emmental cheese. The milk that comes from these ecological areas is characterised by a superior quality of the basic biochemical compounds and their ratio in addition to their rich content in biologically active substances and probiotic bacteria (especially lactic and propionic bacteria), all of these factors having a major impact on the process of obtaining and valorising the different types of Emmental cheese. The quality of the Emmental cheese is also ensured by the temperature-humidity regime that is specific to the mountainous region together with the addition of sodium chloride and the action of specific mechanical factors that help with the process of whey extraction. There are many types of Emmental cheese that differ based on the technological process, time of maturation and the specific of the region where it is made.

Key words*: biodiversity, cheese, Emmental cheese, milk bioactivity, mountain pastures.*

INTRODUCTION

The globalisation of food market has flourished in the last decades resulting in an important diversification and broadening of Emmental types of cheese. This situation has led to the exponential rise of commerce of food in the European community territory and thus, all over the world (Ssilvanikove et al., 2015). Globalisation represented a big challenge for milk processors that had to continuously raise the quality and security of the marketed products and also satisfy the diversified demands of the consumers (Svederberg et al., 2012). According to recent data, milk and dairy products represent a big proportion of the average Europeans' diet. Milk and dairy products constitute an important source of high quality nutrients (proteins, fats, carbohydrates) and biologically active molecules (enzymes, vitamins, minerals) (Visioli et al., 2014). Current technologies ensure the production of numerous cheese assortments that correspond to the organoleptic, physicochemical and microbiological characteristics needed in order to satisfy the needs and preferences of consumers. According to research conducted in the field, it is completely relevant to categorise dairy products into conventional products that have been prepared using conventional technologies and recipes, traditional products that have been prepared using recipes that are specific to some geographical areas and organic products, that have been obtained using milk that has been certified as organic (Ercolini et al., 2003). The same classification is applied to Emmental cheese. Different types of Emmental cheese (generic and traditional) are produced in large quantities in many European countries such as France, Austria, Germany, Finland and Ireland (Basig et al., 2010). Emmental cheese has its origin in the region with the same name situated in the Berna region of Switzerland, where it has been made ever since the XIIth century, its existence being firstly documented in 1542. In 2002, Emmental cheese acquired

protected designation of origin certificate (Basig et al., 2010). In order to make Emmental cheese, raw milk from mountain pastures is required, where cow feed doesn't consist of silo and the milk is processed in the first 24 hours after milking (Berdague et al., 1990), in copper cauldrons (Rodrighez et al., 2011). Lately there has been a major interest for cheese that has been prepared with raw milk which comes from cows that graze mountain pastures, because it is considered by consumers to have more flavour (Martin et al., 2005). An important characteristic of Emmental cheese is considered to be its unique sensorial perception that consumers prefer, such as the taste, aroma, texture and colour (Spence et al., 2015). The production of raw milk cheese is on the rise because of the high demand of consumers, especially regarding traditional products (Laurenčík et al., 2008). This explains why the normal microflora of raw milk constitutes an essential factor in the production, fabrication and maturation of traditional cheese (Beresford et al., 2001).

THE SPREAD AND PERSPECTIVE OF MOUNTAIN PASTURES

Mountain areas, especially the ones situated over 800 m altitude, present alongside the numerous advantages, several disadvantages. These are represented by the limited possibilities of proper utilisation of mountain grazes and terrains due to the difficult climate conditions and also because of the rough terrain that does not allow the use of farm equipment (Coppa et al., 2019). Therefore, wild pastures represent the only possibility of using agricultural terrains in high altitude regions. In the European Union, the limit of mountain regions start at minimum 600-700 m elevation over the sea levels. It is important to mention the fact that the levels of altitude can vary depending on the country, climate and the elevation of the geographical area (Coppa et al., 2019). Currently, it is estimated that the pastures area in the European Union represent 18.5% out of the usable agricultural land (Coppa et al., 2011). Alpine pastures make up 17.8% of the total usable agricultural land of the European Union and the cattle grazing on those pastures account for 10.5% of the total of dairy cattle in the EU (Santini și col., 2013). Production costs are higher in mountain areas because of the lower profitability of dairy cattle farms (Santini et al., 2013; Martin et al., 2014). This impediment can be overcome by the reduction of costs regarding the production and collection of the milk through commercial strategies to raise the prices of the dairy products that can be certified as products with protected designation of origin (Coppa et al., 2019). The advertisement of products with special sensorial characteristics has a major impact on the gratification of the consumers demand and preferences (Martin et al., 2005; Bentivoglio et al., 2019).

COMPOSITIONAL CHARACTERISTICS OF MOUNTAIN MILK

Milk is considered to be the "perfect food" because it contains all the essential nutrients and vitamins, enzymes and other biologically active molecules. The biochemical composition of milk is as follows: water (85.5- 89.5%), dry matter (10.5-14.5%), fat (2.5- 6.0%), protein (2.9-5.0%), lactose (3.6-5.5%), minerals (0.6-0.9%), vitamins (Asadullah et al., 2010). Water is found in the biggest proportion in whey (70-90%) and it helps stabilize the other constituents by assuring the dispersion of casein micelles, fat emulsification during the processing and bacterial growth (Guinee and O'Brien, 2010). Casein proteins represent approximately 80% of milk protein and they coexist amongst insoluble minerals like calcium phosphate and together with fat they make up the solid faction of the milk, known as rennet. Milk also has a liquid fraction represented by the whey, which is made up of water, lactose, lactic soluble proteins, citric acid, some minerals, enzymes, free amino acids and peptides (Guinee and O'Brien, 2010). During the process of cheese making, milk is partially dehydrated by the controlled expulsion of whey and partially of fat, casein and some minerals (Guinee and O'Brien, 2010). The processes of casein aggregation and dehydration ensure the quality of cheese as finite products (Guinee and O'Brien, 2010). There are four types of casein (α s1, α s2, β and k), that are found in different proportions in the total casein count (38%, 10%, 35%, 15%)

(Jerónimo et al., 2016). Through the process of casein combination, micelles are formed and they are part of the cheese structure. Casein micelles are colloidal particles that have a spherical shape and a diameter of approximately 40-300 nm (McMahon and Oommen, 2008).

Whey protein (0,6-0,7%) are represented by β lactoglobulines 54%), α lactalbumine (21%-metal protein that is bonded by Ca2+), immunoglobulins (14% - IgG1, IgG2, IgA, IgM) (Guinee et al., 2010) and lactoferrin (O'Callaghan et al., 2019). Lactic enzymes are very important as well, especially in the evaluation of the hygienic quality of milk. Milk has an important natural catalytic activity which is a useful indicator of a possible microbial contamination of the mammary gland and of milk itself (Maubois, 2018). Milk lipids can have significant variations (3-5%) depending on the breed, general health status, mammary glands and feed. Milk fat includes triglycerides (96-99%), phospholipids (0,8%), diglyceride (0,3%), sterols, some free fatty acids, carotenoids and fat-soluble vitamins (Huppertz et al., 2009). Milk fat is distributed as dispersed globules that are surrounded by a lipoprotein membrane (Wiking et al., 2004). The membrane has the role of protecting and stabilising the fat globules against turbulence and fusion, and also against microorganisms like *Pseudomonas spp*. (Ward și col., 2006). The deterioration of the membrane of fat globules during the manipulation of the milk leads to the accumulation of free fat in the milk, then in the cheese, that is responsible of undesirable taste (sour, metallic, soapy), which can be specific to hard and semi-hard cheese (Emmental, Gouda, Cheddar) (Deeth, 2006).

THE INFLUENCE OF MOUNTAIN PASTURES ON THE BIOLOGICALLY ACTIVE COMPONENTS OF MILK

Carotenoids (lutein and carotene) can be considered potential biomarkers and their concentrations must be monitored in milk and various dairy products (Nozière et al., 2006). Carotenoids are found in large quantities in cow milk and they have a big impact on the nutrition and general health status of humans. They work as natural antioxidants and precursors of vitamin A. The lactic concentration of carotenoids can present important variations, mainly due to season, sometimes making up to 50% (Agabriel et al., 2007). Carotenoids are liposoluble, so they easily transfer from milk to cheese (Nozière et al., 2006), their concentrations correlating with the yellow richness of cheese (Kilcawley, 2018).

Terpenes are one of the bioactive components of milk that according to Agabriel et al. (2007), are found in higher concentrations in mountain milk than in milk that has other provenance. Other studies suggest that terpenes that are found in milk and cheese can reach high levels when the cows graze wild pastures, rich in plants from the *Apiaceous* family, thus explaining a more intense smell of the milk compared to the milk that comes from cattle, predominantly fed with *Gramineae* (Kilcawley et al., 2018). The terpene content of mountain pastures plants varies depending on the botanical family they belong to (Prache, 2009; Tornambé et al., 2006). Other researchers also found large quantities of sesquiterpenes and monoterpenes in the plants that are specific to mountain pastures (Engel et al., 2007). It is important to mention the findings of De Noni et al. (2008) regarding the terpene profile of mountain milk, which is dominated by monoterpenes, the same as in cheese made from this type of milk. It has been proven the fact that terpenes go through biotransformation in the rumen and large intestine without hepatic or renal excretion when their concentration exceed their normal levels (Poulopoulou et al., 2012). Even though data suggests there is a slight passing of terpenes found in plants in the milk and cheese, there is still a need for more research in order to confirm this hypothesis. Belviso et al. (2011) discovered other terpenes in dairy products that aren't found in the feed. The same authors highlighted the biosynthetic and metabolization potential of terpenes in the rumen. Lactic bacteria can modify and synthetize terpenes that reach the milk and consequently cheese, after ruminal metabolization.

Volatile compounds. Natural pastures, especially mountain pastures, give Emmental cheese a distinct aroma through the over 200 volatile compounds that are found in the greenery (Taylor et al., 2013). Volatile

compounds are the result of proteolysis, lipolysis and metabolization of lactose, citrate and microorganisms during the maturation phase (Taylor et al., 2013). Volatile compounds make up several groups of non-terpenoid products (lactones, acids, esters, fenoles, aldehydes, alcohols, sulphide compounds) that are found in the pastures and they can contribute to the milk flavour (Kilcawley et al., 2018). The cheese flavour is more complex because of the activity of numerous microorganisms that are later added (lactic bacteria, yeasts and moulds). The concentrations of volatile compounds rise during the fermentation and maturation phases of the cheese (Kilcawley et al., 2018). As it was mentioned before, the high concentration of fatty acids and polyunsaturated fats in mountain milk can oxidase and generate lipid peroxides, aldehydes, ketones and alcohols (Havemose et al., 2006). The same author states that the sources of many aldehydes and ketones are extremely hard to identify because they come either directly from feed or from the maturation phase of the cheese. Stefanon et al. (2004) analysed cheese made from the milk of cattle which were fed with silage and mountain grass and they found a higher concentration of volatile alcohols than the concentration resulted from consumption of corn, corn silage or hay from lowland. It is important to also note the results obtained by Bugaud et al. (2011) that show the fact that cheese made from cattle mainly fed with hay contains larger quantities of 2-methyl-butanol and 3-methylbutane. Other compounds that contribute to the flavour of milk and cheese are simple carboxylic acids that result from lipolysis and the metabolism of amino acids and carbohydrates (Kilcawley et al., 2018). The highest concentrations of fatty acids are found in cheese made from mountain milk (Falchero et al., 2010). There are numerous studies regarding pastures, milk and cheese from mountain areas that emphasise the rich content of fatty acids depending on the assortment and the age of the cheese or on the transfer of fatty acids in the milk either by ingestion or inhalation. Sulphide volatile compounds are potentially aromatic compounds because of the smell and their intense flavour (Falchero et al., 2010). According to research studies, toluene can be a potential biomarker for milk that comes from cattle that graze natural pastures (O'Callaghan et al., 2016) because it is a product of ruminal degradation of carotene (Villeneuve et al., 2013). The highest levels of carotene can be found in pasture grass and other fresh feed (Coppa et al., 2011). Some authors state that toluene is largely present in cow milk, which explains its high concentrations in cheese made from milk that comes from cattle that graze natural pastures (Cornu et al., 2009). Toluene is considered to have numerous sensorial influences such as sweet, nutty, almond-like (O'Callaghan et al., 2017) pungent, etheric, fruity or rancid (Coppa et al., 2011).

PRINCIPLES OF PROCESSING ADAPTED TO THE SPECIFIC OF CHEESE MADE IN MOUNTAIN AREAS

Cheese production includes the jellification of milk and the dehydration of the gel in order to form the cheese. The cheese making process is based on the destabilisation of the micelle structure of casein through the alteration of the physico-chemical properties of the solution (O'Callaghan et al., 2019). After the primary processing (stirring, texturization, pouring and pressing), cheese is poured in different shapes (that range from small quantities to 100-120 kg). After the preparation of the cheese, it can be consumed fresh in the first week after it was made or it can be matured for longer periods of time (starting from 2 weeks up to 3-6 months, even up to a year or two) like Emmental cheese (Guinee et al., 2010). In order to make most cheese it is mandatory to add rennet that contains chymosin, an enzyme capable to hydrolyse the k casein (Fagan et al., 2017). This process will determine the destabilisation of the casein micelles and it will lead to the coagulation of milk under the form of a gel that represents the actual cheese (Fagan și col 2017).

The coagulation process represents an important step in the fabrication of cheese because it marks the passing of milk from the liquid state to solid state (Gassi et al., 2017). There are two procedures used for milk coagulation namely the lactic way (acidification) and the enzymatic pathway through the addition of ferments in the milk.

The enzymatic pathway consists in the addition of rennet in the mildly acidified milk and it is specific to pressed cheese including Emmental cheese (Gassi et al., 2017). The two procedures are often combined in cheese making technologies. The acidity is due to the lactic bacteria that transform lactic acid. This process is essential for milk coagulation and it requires a minimal temperature threshold and it varies according to the cheese assortment (Fox and McSweeney, 2017). Acidophilic bacteria from milk can be either mesophilic or thermophilic. Mesophilic rod-shaped bacteria are used in order to obtain raw milk cheese (processed at temperatures below 40°C) and for the formation of the rind of the cheese. In contrast, thermophilic bacteria are used in high temperature processes (over 50°C) to obtain boiled cheese like Emmental (Fox and McSweeney, 2017). Mesophilic bacteria grow at temperatures between 25-40°C (Fröhlich-Wyder et al., 2017) and they consist of *Lactococcus* spp. and *Leuconostoc* spp. (Hayaloglu, 2016). Various bacteria are used in the cheese making process like *Lactococcus lactis, Lactococcus lactis* subsp. *cremoris, Lactobacillus paracasei, Lactobacillus rhamnosus, Leuconostoc* spp. and *Staphylococcus* spp. (Cotter et al., 2017). *Lactococcus* spp. are essential because they transform lactic acid in glucose and so they are considered to be homofermentative (Fröhlich-Wyder et al., 2017). *Leuconostoc* spp also have essential properties because they gather heterofermentative bacteria involved in the metabolization of lactose in lactate, ethanol and CO_2, which are responsible for the holes in the Emmental cheese (Fröhlich-Wyder et al., 2017). It is important to mention the fact that these bacteria withstand low pH levels, which is an advantage at the end of the acidifying process. Among thermophile bacteria, there are *Streptococus termophilus, Lactobacillus helveticus* and *Lactobacillus delbrueckii* ssp. *lactis* (Fröhlich-Wyder et al., 2017). The rennet is a mixture of animal enzymes used to make cheese (Andrén, 2011). After 1980, the recombinant DNA technology allowed the creation of chymosin through gene cloning of a protein making gene in *Escherichia coli* (Uniacke-Lowe and Fox, 2017). Rennet has been used in cheese production for thousands of years; its traditional form was a mixture of chymosin and pepsin enzymes secreted in the abomasum (Uniacke-Lowe and Fox, 2017). These two enzymes have a proteolytic activity that is the basis of the coagulation process, jellification being the selective hydrolysis of casein in phenylalanine-methionine. This peptide bond is caused by the addition of the rennet and the acidifying to 20-40°C and a pH around 4.6 (Andrén, 2011). The calcium ions play an important part in milk coagulation by inducing casein micelle aggregation and the coagulation of the casein (Guinee and O'Callaghan, 2013). Milk ionized calcium is in an equilibrium with the casein calcium with a direct influence over the aggregation and jellification of the milk (Guinee and O'Callaghan, 2013). Also by the addition of calcium chloride in the milk, a soft, elastic texture is produced, which is essential for the formation of holes that are specific to Emmental cheese (Fröhlich-Wyder et al., 2017). The milk clot contracts and removes the whey in the presence of the rennet, proper acidity and optimal temperature (Gassi et al., 2017). The resulted gel is ten times more concentrated in casein, fat and calcium phosphate than milk. The resulted product is processed in order to help remove the whey by cutting it into cubes (of approximately 0.5-1.5 cm in diameter), stirring, rising the temperature and reducing the pH levels in order to ferment lactose in lactic acid (Lucey et al., 2003). After the whey is drained, small particles of cheese are formed and they make up a coagulated mass that is put in the desired mould, it is pressed and then the salt is added, depending on the type of cheese that is produced (Lucey et al., 2003). Traditionally Emmental cheese is made in copper cauldrons. The propionic fermentation in the Emmental production is a unique phenomenon. It is well-known that propionic bacteria that can be found in raw milk can cause problems during the maturation phase. Copper slows down the explosive fermentation of propionic acid and inhibits spores growth like *Clostridium tyrobutyricum*, thus reducing the risk of the cheese blowing during the later stage of maturation (Rodriguez and Alatossava, 2010). Copper forms bonds with the sulphide compounds resulted from the

metabolism of amino acids which leads to the nutty sweet taste of the cheese.

One of the main conditions for propionic acid to form and to later stimulate CO_2 to create the specific holes, is to add water to the milk or into the cheese. For Emmental cheese, 12-20% water is added in order to reduce the lactose concentration and to rise the pH (up to 5.2-5.35) and accelerate the formation of propionic acid (Fröhlich-Wyder et al., 2017). Studies suggested that the formation of the holes during maturation is due to some microparticles that through their capillary structures are found in plant tissue and they are most likely the natural precursors of some nuclei that generate the holes during propionic acid fermentation (Guggisberg et al., 2015). Another particularity of the formation of the holes is the fact that during maturation, the propionic bacteria consume the lactic acid and it releases CO_2 and the resulted gas makes the specific holes in Emmental cheese. This type of cheese is industrially produced with pasteurized milk in countries all over the world and the process is controlled by enzymes and perfected lactic cultures in order to obtain similar qualities with the original. The addition of salt is a very important step in the cheese making process. It has both a conservational role and it also gives it flavour (Guinee and Fox, 2017). Salting can be accomplished either through dipping the cheese in brine for a certain period of time or by searing the cheese with fine or coarse salt or both, all of this depending on the type of cheese (Guinee and Fox, 2017). The success of the maturation phase depends on following the guidelines of temperature and humidity in the storage room, their values depending on the type of cheese being made.

CONCLUSIONS

The gathered data argue the effects of mountain areas on the composition of milk and cheese, especially Emmental cheese, that are differentiated by the higher quality assured by the usage of milk from cattle raised in mountain conditions. The flora of mountain pastures is characterised by the bioactive compounds rich in terpenes, carotenoids, propionic bacteria and volatile compounds that can be found in the raw milk and dairy products. All of these mostly assure the nutritious, bioactive and sensorial qualities of Emmental cheese that is considered to be the "king" of all assortments of cheese. The fermentation process is also potentiated by the enzymatic content of the mountain milk that is biodiverse because of the mountain pastures and also because of the quality of the rennet and added mesophilic lactic bacteria. An important part in the processing of milk that is used to make Emmental cheese is the use of copper cauldrons that neutralise pathogen microorganisms and slow down the explosive fermentation of propionic bacteria thus preventing cheese defects. Propionic bacteria will stimulate the normal fermentation that will release CO_2 that is essential in order for the specific holes to form. The water that is added during the process reduces the lactose concentration and helps the pH levels rise consequently assuring the optimal conditions for propionic fermentation and release of CO_2. In order for the Emmental cheese making process to be success, it is important to strictly obey the temperature regulations during the coagulation phase and also during the pressing, salting and maturation phases. The specific conditions that need to be followed during the maturation phase are the temperature and humidity, the process lasting from a few weeks up to 12-24 months depending on the tradition of the region. Generally mountain cheese and other dairy products present attractive sensorial profiles compared to lowland products.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

THE INFLUENCE OF THE STRUCTURE OF THE DAIRY COW RATION ON CO_2 EMISSIONS

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Abstract

The aim of the research undertaken was to highlight that emission reductions can be made available to producers in the steer farming sector and the adoption of current best practices and technologies for the rearing and health of animals, feed rations can be a tool that would help the dragline sector reduce greenhouse gases, and was realized on the Moara Domneasca farm on a flock of 27 dairy cows at different stages of Montbeliarde's lactation between November 2019 and September 2020. Daily milk production was establised per lactation cycle, within the lactation cycle of 3 distinct stages and the establishment of two seasons, summer and winter. The influence of feed strategies applied on milk production, manure chemical composition and CH_4 and CO_2 emissions were analyzed. The dairy production of the cows varied in the management of green feed rations between 21.97 L/head in the upward stage of lactation and 20.54 L/head in the plateau phase and in winter succulent rations, milk production was 21.29 L/head in the ascending phase, 19.93-20.27 L/head in the plateau phase and 18.56 L/head in the ascending phase. The methane emission from enteric fermentation has the highest values for the variants 6 and 4, which contains rots (sunflower and soybean), maize, and wheat bran and the lowest emissions are recorded for the ration variant 1 which is rich in green fodder.

Key words*: emissions, enteric fermentation, manure, milk production.*

INTRODUCTION

Naturally occurring methane is generated by anaerobic fermentation, where bacteria break down organic matter producing hydrogen (H_2), carbon dioxide (CO_2) and methane (CH_4). This process naturally occurs in the digestive system of domesticated and wild ruminants, natural wetlands, and rice patties. In ruminants, methane is produced mostly by enteric fermentation where microbes decompose and ferment plant materials, such as celluloses, fiber, starches, and sugars, in their digestive tract or rumen. Enteric methane is one by-product of this digestive process and is expelled by the animal through burping. While other by-products (acetate, propionate and butyrate) are absorbed by the animal and used as energy precursors to produce milk, meat and wool. Enteric methane production is directly related to the level of intake, the type and quality of feed, the amount of energy consumed, animal size, growth rate, level of production, and environmental temperature. Between 2 to 12% of a ruminant's energy intake is typically lost through the enteric fermentation process. (www.fao.org).

Under normal feed conditions, methane accounts for 15-30% of the total ruminal gas (mixture of carbon dioxide, methane, hydrogen nitrogen, etc.). The proportion of these gases is variable according to the nature of the feed and the intensity of the fermentation. The production of light methane is not directly proportional to the digestibility of the feed consumed. Highly digestible feed forms less methane per unit of calorific energy consumed than those with lower digestibility (Blaxter & Clapperton, 2007).

MATERIALS AND METHODS

The research was carried out on the Moara Domneasca teaching farm of the University of Agronomic Sciences and Veterinary Medicine of Bucharest, on a flock of 27 dairy cows at different stages of Montbeliarde's lactation between November 2019 and September 2020.

The dairy cows are kept in free stabulation in a shelter with modern facilities. During the summer or when the weather is favorable, the animals are taken into the enclosure. For the rational feeding of this category of animal, knowledge should be given of the energy and nutrient requirements so as to formulate balanced rations, but also to reduce polluting emissions from farms.

The dairy cows are kept in free stabulation in a shelter with modern facilities. During the summer or when the weather is favorable, the animals are taken into the enclosure. For the formulation, optimization and verification of feed rations for dairy cows account has been taken of their average body weight (650 kg), daily milk production per lactation cycle, the establishment within the lactation cycle of 3 distinct stages and the establishment of two seasons specific to our country, namely summer and winter.

The bottom-up phase of the lactation cycle runs from the birth to the maximum daily yield, which is common in the first two months of lactation. The increase in milk at this stage is due to the multiplication of the alveolar tissue in the ug and the establishment of a new hormonal balance characteristic of lactation. The ingestion capacity is at a level of 85-90% of the maximum value. Specific problems are caused by reduced ingestion capacity, requiring the use of high-quality high energy and protein feed (Maciuc, 2015).

The plateau phase is characterized by the relatively constant maintenance of milk production and lasts 5-7 weeks. During the plateau phase, the hormonal balance is maintained at a favorable level to the intense synthesis of milk, characterized by a milk production level of about 96 to 98% of the maximum daily yield, while the ingestion capacity is about 90 to 95% of the maximum value. At this stage, the mobilization of body reserves is insignificant, which implies sharp increases in the energy inputs of the ration at the same level of milk production in week 3 of lactation.

Specific problems are caused by reduced ingestion capacity compared to requirements, requiring the use of high energy and protein high quality feed. From a practical point of view, it is the most complex stage and it has to be given special attention (Popescu et al., 2005). Failure to ensure nutrient requirements in the ration over a relatively short period of a few days leads to large reductions in milk production over a long period of time.

The downward phase of the lactation curve means the drop in milk production, initially slow and then growing more pronounced, until weaning. The normal rate of drop in milk production is considered to be 10% per month for pregnant cows and 4-6% per month for non-increase in the residual fraction of the milk, the involution of the glandular tissue of the udder and the state of gestation (Miresan et al., 2003).

In the first part of this phase, the feeding of cows is relatively easy to ensure due to the high ingestion capacity and the continuous decline in milk production. The nutrient to be administered may also be of medium quality, as the nutrient intake can be satisfied by higher food consumption. At the end of lactation there is a process of total recovery of body reserves, which implies greater increases in the energy and protein inputs of the ration intended for this purpose. At this stage, there are no specific problems in the preparation of the ration. From a practical point of view, it is the most manageable stage due to the high ingestion capacity compared to the level of milk production. The use of the test chemical in the food is recommended. Starting with the 40^{th} lactation week, attention will also be paid to the intake of nutrients for gestation (Pop et al., 2006).

Feed and by-products of plant origin have been introduced in the construction and optimization of rations in order to highlight the possibility of developing farms where very good results are achieved only by using local feed resources wisely.

A particularly important role during the summer period is the green mass, which can cover 60-80% of the volume feed content of the ration and constitutes a food rich in the nutrients needed for milk production.

The feed ration is relatively balanced, except for the increased protein and mineral feed in the event of the administration of higher amounts of green fodder during the summer period.If the green mass quality is very good, the quantities of concentrates in the ration may be reduced by 5-10%.

During the winter period the green table is replaced by succulent soiled fodder without significantly altering the intake of concentrates in the ration.

RESULTS AND DISCUSSIONS

The influence of feed strategies applied on milk production.

The evaluation of the productive performance (Table 1) was carried out with a milk quantity and quality monitoring system, which enabled the identification of the animals in the milking room, the monitoring of breeding and ruminants (DairyPlan C21).

The dairy production of the cows varied in the management of green feed rations between 21.97 L/head in the upward stage of lactation and 20.54 L/head in the plateau phase. In the case of winter succulent rations, milk production was 21.29 L/head in the ascending phase, 19.93-20.27 L/head in the plateau phase and 18.56 L/head in the ascending phase. Romania's entry into the EU has imposed the common market milk quality standards.

In terms of milk fat content, it varied between 3.62-3.78% in the upward phase of the lactating curve, 3.77-3.91% in the plateau phase (3.88-3.91% in winter rations and 3.77% in summer ration) and 3.54% in the downward phase of lactation.

Milk protein content varied between 3.17-3.23% in the upward phase of lactation, 3.27-3.39% (3.30% in summer ration) in the plateau phase and 3.08% in the descending phase. The average acidity of the milk was 6.50 and all milk samples were in line with the recommendations on the maximum limit of the total plate count (10 x 10^4 NTG/ml), the average being 8.68 x 10^4 NTG/ml.

The influence of feed strategies applied on manure chemical composition

In order to determine the influence of the nutrient variants tested on the chemical composition of the manure (Table 2), three samples per ration variant have been analyzed.

It is noted that when using green feed rations the proportion of water in the manure determined by drying in the oven at 105°C varied between 76.36 to 77.12%, while for the other rations the water content was 70.11 to 75.07%. The ash content determined by ashing at a temperature of 50°C varied between 8.01% on the green fodder variant in the ration and 10.51% on the variant of the ration administered in the descending phase of the lactation curve.

The nitrogen content determined by the Kjeldahl method was lower for variations in green fodder ration in the structure (0.45-0.55%) and for the other variants the nitrogen values were between 0.60-0.87%.

The influence of feed strategies applied on shelter's methan emissions

Methane emission from enteric fermentation will be estimated using IPCC method 2. The calculation of the methane emission shall be carried out on the basis of equations 10.19, 10.20, 10.21 from *IPCC Good Practice Guidance and Uncertainty Management in National Greenhouse Gas Inventories, 2006:*

$$Emisions = EF_{(T)} * \frac{N_{(T)}}{10^6}$$

where:

Emisions = methane emission from enteric fermentation, kg CH_4/year;

$EF_{(T)}$ = emissions factor for dairy cow, kg CH_4/head/year

$N_{(T)}$ = the herd of animals of the species/ category T

T = category of animal

$$\text{Total } CH_{4\ ENTERIC} = \sum_i E_i$$

where:

Total $CH_{4Enteric}$ = total methane emissions from enteric fermentation, kg CH_4/year

E_i = emissions from animal categories

$$EF = \left[\frac{EB*\left(\frac{Ym}{100}\right)*365}{55.65}\right]$$

where:

EF = emission factor, kg CH_4/head/year;

EB = gross energy, MJ /head/year;

Ym = methane conversion factor, which is the percentage of raw energy in the administered feed converted to methane

55.65 (MJ/kg CH_4) = energy content of CH_4

Gross energy (GE)

The following equivalences (Stoica, 2001) have been used to calculate the calority of the gross energy of each ration: 1 g crude protein (PB) = 5.72 kcal; 1 g raw fat (GB) = 9.5 kcal; 1 g crude fiber (CelB) = 4.79 kcal; 1 g SEN (non-

nitrogenous extractive substances) = 4.17 kcal. The formula for calculating GE is:

GE (kcal/kg) = 5.72·PB + 9.5·GB + 4.79·CelB + 4.17·SEN

where:
GE = gross energy intake (kcal/kg);
PB = crude protein (%);
GB = raw fat (%);
CelB = crude fiber (%);
SEN = non-nitrogenous extractive substances (%).

The rations have been formulated earlier according to the animal feeding schedule and the values of crude protein, crude fat, crude cellulose and non-nitrogenous extractable substances (Table 2) have been obtained from analyzes carried out in its own laboratory, i.e. by calculation (SEN).

The values of gross energy (MJ/kg) for feed constituents of the rations and the total energy value of the rations delivered to the dairy cows expressed in GE are given in Table 3.

Digestible energy (DE) is used to express the nutritional value of feeding stuffs and rations, especially for grazing animals. Mathematical equations have been used to establish it by calculation, as in the case of raw energy, but in this case the digestibility content of nutrients is taken into account, taking into account the digestibility factors specific to each feed and species, namely taurine (Dragotoiu et al., 2017), then multiplied by the energy equivalents for digestible energy, which are different by species. The percentage of digestible energy (ED%) in the raw energy is calculated by applying the three simple rule according to the relationship: ED % = (ED/EB)·x 100.

The following equation shall be used to calculate the values of Ym: Ym = -0.0038 x (ED%)2 + 0.3501 x ED% – 0.811 (Cambra-Lopez equation, 2008).

The equation for calculating the enthic CO_2 emission shall be (Users' guide for estimating carbon dioxide, methane, and nitrous oxide emissions from agriculture using the State inventory tool, 2019):

CO_2 enteric (kg/year) = (Emission CH_4 x 25 GWP) / 1,000,000,000

The values obtained for the methane emission from enteric fermentation and the CO_2 equivalent are given in Table 4, Figures 1 and 2, respectively.

Table 1. The quantitative, qualitative and microbiological parameters of milk production

Exp. variant	Lactation phase	Milk production (l)	Milk protein (%)	Milk fat (%)	pH	Total number of germs (NTG/ml x 10^4)
V1	The upward phase	21.97±0.55	3.17±0.04	3.62±0.37	6.47±0.04	8.8±0.16
V2	The upward phase	21.29±1.26	3.23±0.21	3.78±0.52	6.50±0.02	8.5±0.09
V3	Plateau phase	20.27±0.84	3.27±0.07	3.88±0.42	6.48±0.05	8.7±0.08
V4	Plateau phase	19.93±1.26	3.39±0.07	3.91±0.73	6.53±0.04	8.6±0.08
V5	Plateau phase	20.54±0.84	3.30±0.07	3.77±0.42	6.48±0.05	8.9±0.12
V6	Down phase	18.56±0.88	3.08±0.10	3.54±0.41	6.52±0.03	8.6±0.11

Table 2. Chemical composition of manure obtained in experimental period

Feed variant	Water (%)	Ash (%)	N (%)
V1	77.12	8.01	0.45
V2	75.07	8.24	0.60
V3	75.25	8.52	0.75
V4	73.42	9.56	0.81
V5	76.36	8.35	0.55
V6	70.11	10.51	0.87

Table 3. Value of gross energy (GE) of the component feeding stuffs and rations delivered to cows during the experimental period

Feed	GE (Mj/kg)	DE (Mj/kg)	Total ration GE (Mj)	Total ration DE (Mj)	Ration variant
Lucerne hay	16.25	8.26	370.68	269.17	V1
Hay clover	15.69	7.86	333.90	216.61	V2
Fodder beet	2.37	1.87	314.81	205.16	V3
Beer Brewery	3.78	2.35	322.16	205.34	V4
Corn soiled	4.78	2.78	329.51	218.68	V5
Spring bowl	3.36	2.17	315.52	198.20	V6
Clover	3.55	2.34			
Maize	16.57	14.20			
Barley	16.14	13.05			
Sunflower rot	17.88	12.56			
Soybean rot	17.87	16.05			
Wheat bran	16.57	10.69			

Table 4. CH_4 emission from enteric fermentation

Experimental variant	GE (Mj/zi)	DE (Mj/zi)	DE(%)	Ym	EF	Head number	CH_4 emissions (kg/year)	CO_2 x 10^{-9} Emissions (t/year)
1	370.68	269.17	72.615	4.574	111.21	10	1112	27.803
2	333.9	216.61	64.873	5.909	129.40	10	1294	32.350
3	314.81	205.16	65.169	5.866	121.12	12	1453	36.336
4	322.16	205.34	63.739	6.066	128.17	12	1538	38.452
5	329.51	218.68	66.365	5.687	122.91	10	1229	30.727
6	315.52	198.20	62.817	6.187	128.03	12	1536	38.408
							8163	204.077

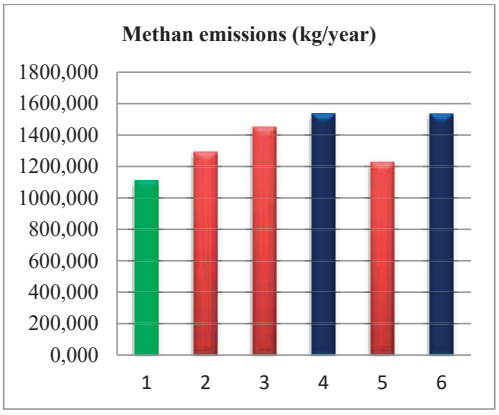

Figure 1. Methane emissions from enteric fermentation in the 6 variants

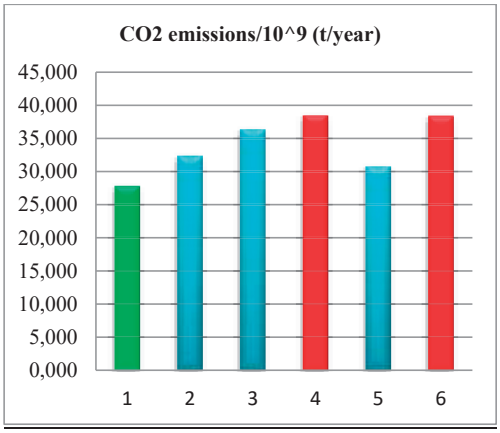

Figure 2. CO_2 equivalent emissions in the 6 variants

From the analysis of the data presented in Table 4, it can be observed that the methane emission from enteric fermentation has the highest values for the variants 6 and 4, the values being close (1538 kg/year and 1536 kg/year respectively) and the lowest emissions are recorded for the ration variant 1 with green fodder. In variant 2 with a reduced proportion of fibrous fodder a value of 1294 kg/year has been obtained.

The trend of equivalent CO_2 emissions also closely follows the line of CH_4 emissions from enteric fermentation and is directly dependant. Sun & Colabas (2012) reported substantially lower CH_4 values in sheep fed with green fodder and rape (*Brassica napus*) respectively. Dewhurst (2012) provided an overview of the various aspects of feeding lactating cows with contaminated feeding stuffs (corn, pulses).On the basis of these comments, it concluded that

the decrease in fiber content and the faster passage of pulses through the digestive tract of the cows decrease the production of CH_4.

CONCLUSIONS

Together with the aspects of milk production, a number of measures are needed on the use of feed and feeding techniques that take into account the digestibility, quality and composition of the feed ration, which can reduce the methane generated during digestion.

The methane emission from enteric fermentation has the highest values for the variants 6 and 4, which contains rots (sunflower and soybean), maize, and wheat bran and the lowest emissions are recorded for the ration variant 1 which is rich in green fodder. In variant 2 with a reduced proportion of fibrous fodder a value of 1294 kg/year has been obtained. It is a middle value of CH_4 emissions from enteric fermentation.

Emission reductions can be made available to producers in the steer farming sector and the adoption of current best practices and technologies for the rearing and health of animals, feed rations can be a tool that would help the dragline sector reduce greenhouse gases.

ACKNOWLEDGEMENTS

This research work was financed from Project ADER 9.1.4./2019 “Research on improving the productive efficiency of animals of bovine, ovine, caprine, pig and poultry species, by reducing the total annual emissions of greenhouse gases, expressed in tonnes of CO_2 equivalent”.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

IMPLICATION OF DIETARY PHYTOGENIC MIXTURE IN MODULATING THE INTESTINAL MICROFLORA OF BROILERS RAISED IN THERMONEUTRAL AND HEAT STRESS CONDITIONS

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Abstract

The paper presents the effect of a dietary phytogenic mixture on intestinal microflora of broilers raised under thermoneutral conditions (TN) and heat stress (HS). The feeding trials were conducted on 120 Cobb 500 broilers (60 chicks/trial) raised in environmentally- controlled digestibility cages. Up to 14 days the chicks were fed a conventional diet. On the 14th day, they were divided in four homogeneous groups (2 groups/ trial, 30 chicks/ group). In the first trial, two groups (C-TN and PM-TN) were kept in TN. In the second trial, two other groups (C-HS and PM-HS) were kept in HS (32°C). Both trials used the same structure of diets. Compared with the control diet (C), the experimental diet (PM) included the addition of 1% phytogenic mixture (bilberry leaves, peppermint leaves, fennel leaves and sea buckthorn meal). Both in TN and HS, dietary PM lowered the number of staphylococci and increased the lactobacilli populations in the intestinal and caecal content. E. coli populations have decreased only in the intestinal content of broilers fed PM diet. In conclusion, dietary PM could be an efficient alternative to modulate the intestinal microflora of broilers even in heat stress conditions.

Key words*: broilers, heat stress, microflora, mixture, phytogenic.*

INTRODUCTION

The balance of the intestinal microflora of broilers is crucially affected by exposure to heat stress. This consequence is important because the intestine health is related to the general health of the broiler. In the recent years, many efforts have been redirected for the improvement of intestine microflora. A common strategy for ensuring gut health has been nutritional manipulation. In this regard, many researchers have focused on the use of several dietary supplements, including phytobiotics considering effective stress-alleviating agents (AL-Sagan et al., 2020; Gheorghe et al., 2019; Saracila et al., 2020). They can be included in the diet for broilers as essential oils, powder from different parts of the plant or a combination of plants or oils. In the poultry industry, herbs and herbal products are used to substitute synthetic products to stimulate or promote the development of the chicken gut, ensuring a proper balance between bacterial communities that colonize the intestine, etc.

Bilberry leaves have attracted attention for inclusion in the broiler diet due to the important content of polyphenols, Zn, vitamin E, lutein and zeaxanthin and important antioxidant capacity (Mäkinen et al., 2020; Untea et al., 2020; Varzaru et al., 2020). Popescu et al. (2020) reported that the dietary bilberry leaves positively influence the microbiota of laying hens. Peppermint is a plant with a long history, with strong antibacterial and antioxidant effects in poultry (Abdel-Wareth et al., 2019). The beneficial effects of using peppermint in the broiler diet have included growth promoting efficacy (Toghyani et al., 2010), reduced abdominal fat deposition, and improved antioxidant status (Khempaka et al., 2013). Fennel contains health-promoting volatile essential oil compounds (e.g., fenchone, anethole, myrcene, etc.), amino acids, phenolic compounds, and flavonoids and have been reported to decrease intestinal *E. coli* populations (Ghiasvand et al., 2021), and have had a beneficial impact on growth performance and carcass quality of broilers under heat stress conditions (AL-Sagan

et al., 2020). Sea buckthorn has been studied extensively due to its antioxidant, anti-inflammatory and immunostimulatory properties. Saracila et al. (2020) did not report effects on the coefficients of absorption and performance of broilers fed diet supplemented with sea buckthorn meal and raised under HS.

The aim of this study was to investigate the efficacy of a dietary phytogenic mixture (PM) on the intestinal microflora of broilers raised under thermoneutral conditions (TN) and heat stress (HS).

MATERIALS AND METHODS

The experimental trials were conducted in agreement with the guidelines established by the Ethics Commission of the National Research Development Institute for Biology and Animal Nutrition (IBNA-Balotesti, Romania). Briefly, a total of 120 Cobb 500 broilers were assigned to two feeding trials (for 28 days) with 60 broilers/trial and housed in environmentally- controlled digestibility cages. Until the age of 14 days, a commercial diet (based on corn, gluten and soybean meal) with 22% CP and 3102 kcal/kg ME was administered to broilers. At the age of 14 days, the chicks were weighted and on this criterion were assigned to four homogeneous groups (2 groups/ trial with 30 chicks/group). In the first trial, two groups (C-TN and PM-TN) were raised in thermoneutral (TN) conditions. In the second trial, two other groups (C-HS and PM-HS) were raised in heat stress (HS) conditions (32 ± 1°C). During the experimental period, the light regimen was set to 23h light/ 1h darkness. Feed (mash form) and water were administered ad libitum. Compared to the control diets (C-TN; C-HS) that contained a commercial diet, the experimental diets (PM- TN; PM-HS) included the addition of 1% phytogenic mixture of 40% bilberry leaves, 20% peppermint leaves, 20% fennel leaves and 20% sea buckthorn meal) (Table 1). The mixture is characterized by a high content of polyphenols and a superior antioxidant capacity, as reported by Saracila et al. (2020). The dried and grounded plants used for the mixture were obtained from pharmacies, while sea buckthorn meal was bought from E-Prod SRL, Teleorman, Romania.

Table 1. Diet composition*

Ingredient	Grower stage (14-35 days)		Finisher stage (35-42 days)	
	C	PM	C	PM
Corn	62.00	61.00	60.50	60.00
Soybean meal	26.58	26.58	25.46	25.00
Gluten	4.00	4..00	6.00	6.00
Oil	2.50	2.50	3.75	3.71
Phytogenic mixture (PM)	0	1.00	0	1.00
Calcium carbonate	1.40	1.40	1.33	1.33
Monocalcium phosphate	1.36	1.36	1.13	1.13
Salt	0.37	0.37	0.33	0.33
Methionine	0.26	0.26	0.25	0.25
Lysine	0.48	0.48	0.20	0.20
Choline	0.05	0.05	0.05	0.05
Vitamin-mineral premix*	1.00	1.00	1.00	1.00
Total	100	100	100	100

*1kg premix contains: = 1100000 IU/kg vit. A; 200000 IU/kg vit. D3; 2700 IU/kg vit. E; 300 mg/kg vit. K; 200 mg/kg Vit. B1; 400 mg/kg vit. B2; 1485 mg/kg pantothenic acid; 2700 mg/kg nicotinic acid; 300 mg/kg vit. B6; 4 mg/kg Vit. B7; 100 mg/kg vit. B9; 1.8 mg/kg vit. B12; 2000 mg/kg vit. C; 8000 mg/kg manganese; 8000 mg/kg iron; 500 mg/kg copper; 6000 mg/kg zinc; 37 mg/kg cobalt; 152 mg/kg iodine; 18 mg/kg selenium.

*Diet structure published previously by Saracila et al. (2020)

At 42 days of age, 6 birds per treatment were slaughtered by cervical dislocation. Consequently, the entire intestine was removed from the oesophagus to the cloaca. Samples of intestinal and caecal contents were collected in aseptic medium, in sterilized plastic tubes and stored at -20ºC until bacteriological tests (*Escherichia coli,* staphylococci, lactobacilli, *Salmonella* spp.).

Bacteriological analyses were performed according to the test described by Saracila et al. (2020). Briefly, for the *E. coli* assay, the decimal dilutions (up to 10^{-5}) in lauryl-sulphate medium, were inoculated in 2 Petri dishes on

Levine medium and incubated, and after this the colonies were counted.

The staphylococci assay was performed by immersing the sample in hyper-chlorinated liquid medium, then inoculating in successive dilutions on hyper-chlorinated solid medium and incubated and finally counted. For the lactobacilli method, MRS broth and MRS agar were used as the selective medium and then the forming colonies were counted. *Salmonella* spp. was determined according to SR EN ISO 6579/2003/A1:2007. Scan 300, Interscience (France) was used to count bacterial colonies. The results were reported as a log base 10 colony-forming units (CFU) per gram of intestinal and caecal contents.

Statistical analysis

Data were analysed by 2-way ANOVA using Graph-Pad Prism v. 9.02 (San Diego, CA), with diet (C, E) and temperature (TN, HS) as factors using the following model:

$$Yij = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon ijk$$

Where: y = the dependent variables; μ = the general mean; α_i and β_j = diet and temperature effects; $(\alpha\beta)_{ij}$ = the interaction between diet and temperature; ε_{ij} = the random error. When significant main effects were detected, means were compared using Tukey's multiple range test. Significance was set at a $p<0.05$.

RESULTS AND DISCUSSIONS

Table 2 shows that in HS conditions, dietary PM significantly reduced the number of *E. coli* and staphylococci in the intestinal content. In particular, while staphylococci were significantly lower in the PM-supplemented group than in group C, in TN, *E. coli* populations did not differ between the two groups.

Both diet and temperature significantly influenced the number of *E. coli* and staphylococci populations.

Table 2. Effect of diet supplementation and temperature on intestinal bacterial populations (log10 CFU*/g wet intestinal digesta)

Variable	TN		HS		p-values summary		
	C-TN	PM-TN	C-HS	PM-HS	Diet	Temp.	Diet x temp.
E. coli	6.36[a]	6.35[a]	5.31[b]	5.24[c]	*	***	ns
Staphylococci	6.16[a]	6.15[b]	5.94[c]	5.83[d]	****	****	****
Lactobacilli	7.42[a]	7.43[a]	6.36[b]	6.89[c]	****	****	****
Salmonella spp.	absent	absent	absent	absent	NA	NA	NA

[a, b, c, d]Means in the same column with different superscripts differ significantly ($p<0.05$). Data are presented as mean SEM (n = 6 broilers/group). Asterisks denote statistical significance ($p > 0.1234$ ns, *$p \leq 0.0332$, **$p \leq 0.0021$, ***$p \leq 0.0002$, ****$p<0.0001$).

Interestingly, the lowest populations of *E. coli* and staphylococci were recorded in the intestinal contents of broilers raised under HS. Similarly, in HS the number of lactobacilli was significantly higher in the intestinal content of broilers fed PM diet than in those fed the C diet. If we compare the stress conditions with the normal ones, we see that the lactobacilli have been affected by the applied heat stress, their populations number being smaller.

Ragab et al. (2013) reported a decrease in intestinal pH and the total microflora count of broilers fed a diet supplemented with fennel seeds. The authors explained that these results could be related to the antimicrobial effect of feed seeds. Safaei-Cherehh et al. (2020) showed that the inclusion of 200 ppm fennel extract in broiler (42 days) diet decreased the ileal *E. coli* count due to the antibacterial activity of fennel extract. In a study conducted on Ross broiler chicks, Vase-Khavari et al. (2019) showed that the dietary addition of 0.5% peppermint reduced the total number of *E. coli* and enhanced the caecal populations of lactobacilli.

Vlaicu et al. (2019) reported that a blend of commercial oils (20% rosehip oil, 20% sesame oil, 20% buckthorns oil, 20% nut oil and 20% grapeseed oil) included in the broiler diet (14-42 days) reduced the proliferation of pathogenic bacteria and increased the lactobacilli in the intestine and cecum.

Popescu et al. (2020) explain that dietary bilberry powder influences positively the microbiota by modulating several digestive enzymes that promote the development of lactobacilli and decrease pathogenic bacteria such as *Enterobacteriaceae*.

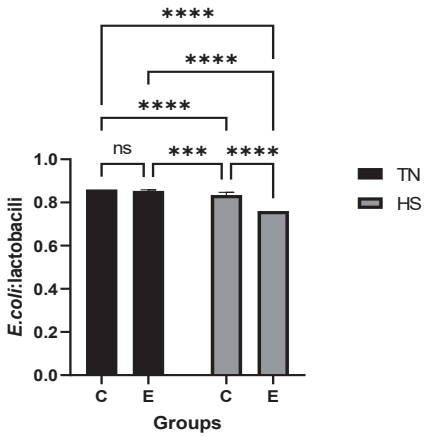

Figure 1. Effect of diet and temperature on *E. coli*: lactobacilli ratio in the intestinal content of broilers

Main effects of diet, temperature, and the interaction are presented in each graph (Prism Graph 9.02). Data are presented as mean SEM (n = 6 broilers/group). Asterisks denote statistical significance (p > 0.1234 ns, *p ≤ 0.0332, **p ≤ 0.0021, ***p≤ 0.0002, ****p<0.0001)

The Figure 1 depicted the influence of dietary PM supplementation and temperature on *E. coli*: lactobacilli ratio. Both temperature and dietary PM supplementation exerted a significant influence on *E. coli*: lactobacilli ratio in the intestinal content.

Under thermoneutral conditions, the ratio was not significant between two groups, but in HS, PM supplementation decreased the ratio. As expected, under HS, the *E. coli*: lactobacilli ratio was lower than that recorded under TN.

The dietary PM supplementation significantly reduced staphylococci populations in caecal content of broilers raised in either TN or HS conditions (Table 3). But dietary PM supplementation had no effect on the intestinal and caecal number of *E. coli*. Regardless of temperature conditions, the number of lactobacilli was significantly higher in groups fed a diet supplemented with PM (PM-TN; PM-HS) than in those fed a diet C (C-TN; C-HS).

Table 3. Effect of diet supplementation and temperature on caecal bacterial populations (log10 CFU*/g wet caecal digesta)

Variable	TN		HS		p-values summary		
	C-TN	PM-TN	C-HS	PM-HS	Diet	Temp.	Diet x temp.
E. coli	10.30	10.16	10.37	10.33	ns	ns	ns
Staphylococci	8.83[a]	8.75[b]	8.65[c]	8.34[d]	****	****	****
Lactobacilli	11.56[a]	11.79[b]	10.66[c]	10.79[d]	****	****	****
Salmonella spp.	absent	absent	absent	absent	NA	NA	NA

[a, b, c, d]Means in the same column with different superscripts differ significantly (p<0.05). Data are presented as mean SEM (n=6 broilers/group). Asterisks denote statistical significance (p > 0.1234 ns, *p ≤ 0.0332, **p ≤ 0.0021, ***p≤ 0.0002, ****p<0.0001).

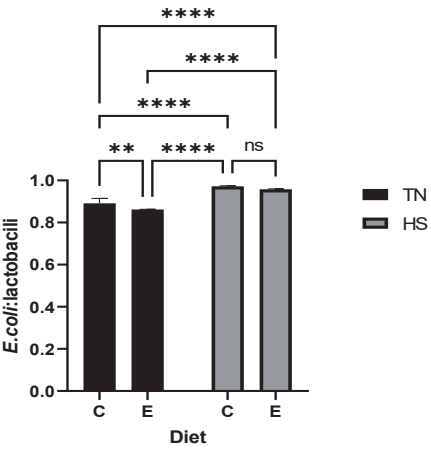

Figure 2. Effect of diet and temperature on *E. coli*: lactobacilli ratio in the caecal content of broilers

Main effects of diet, temperature, and the interaction are presented in each graph (Prism Graph 9.02). Data are presented as mean SEM (n = 6 broilers/group). Asterisks denote statistical significance (p > 0.1234 ns, *p ≤ 0.0332, **p ≤ 0.0021, ***p≤ 0.0002, ****p<0.0001)

The Figure 2 shows that both diet and temperature had a significant influence on the *E. coli:* lactobacilli ratio in the caecal content of broilers. In HS, dietary PM supplementation had no effect on *E. coli:* lactobacilli ratio, while in TN, it showed a significantly lower value. Compared to TN, the values of the *E. coli*: lactobacilli ratio were lower than in HS. This observation confirms the negative effect of HS on the balance of the broiler microflora.

CONCLUSIONS

Dietary PM had a positive effect in reducing *E. coli* populations only in the intestinal contents of broilers subjected to TN and HS. In both HS and TN, the PM diet decreased staphylococci populations and increased lactobacilli in the caecal and intestinal contents of broilers.

ACKNOWLEDGEMENTS

This research work was carried out with the support of Romanian Ministry of Education and Research and also was financed from Project PN 19 09 0102.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

THE BIOCHEMICAL COMPOSITION AND THE FODDER VALUE OF SAND SAINFOIN, *ONOBRYCHIS ARENARIA* (KIT.) DC. IN MOLDOVA

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Abstract

Forage legumes are an essential component of agricultural systems in temperate regions of the world. Sainfoins (Onobrychis Mill.) are Eurasian legume perennial herbs, characterized by the highest condensed tannin content, provide beneficial effects and protect animals against bloat and increase protein absorption. In comparison with other species of fodder legumes, it is less demanding to the soil and if there is enough moisture in the soil, it produces high yields even in the poorest soils. We studied some biological peculiarities, the biochemical composition and the fodder value of a local ecotype of sand sainfoin, Onobrychis arenaria, grown in an experimental field of the National Botanical Garden (Institute), Chişinău. In the second growing season, the sand sainfoin was characterized by optimal growth rate and regenerative capacity after mowing, making it possible to cut it three times per season. It was established that the harvested green mass contained 169.6-264.5 g/kg dry matter. The concentration of nutrients in green mass dry matter was: crude protein 169.6-183.9 g/kg, crude fats 22.0-31.8 g/kg, crude cellulose 273.9-297.7 g/kg, nitrogen free extract 412.8 -447.2 g/kg, soluble sugars 34.4-73.2 g/kg, starch 15.9-23.3 g/kg, ash 74.1-83.6 g/kg, calcium 7.3-11.3 g/kg, phosphorus 2.5-2.7 g/kg, carotene 42.00-45.45 mg/kg. The hay prepared after the first and second cuts contained 163.8-167.9 g/kg crude protein, 16.5-17.0 g/kg crude fats, 315.2-366.7 g/kg crude cellulose, 377.1-413.7 g/kg nitrogen free extract, 75.5-83.6 g/kg ash, 7.7-9.9 g/kg calcium and 2.3-2.9 g/kg phosphorus, 7.86-8.66 MJ/kg metabolizable energy. The biochemical and the fodder value of the prepared haylages were: pH 4.75-5.16, lactic acid 51.5-53.0 g/kg, acetic acid 2.4-4.6 g/kg, butyric acid 0.3-0.4 g/kg, crude protein 164.9-174.9 g/kg, crude fats 26.1-27.4 g/kg, crude cellulose 278.2-300.3 g/kg, nitrogen free extract 428.9-439.3 g/kg, sugars 14.6-25.8 g/kg, starch 16.7-20.7 g/kg, calcium 9.3-9.9 g/kg, phosphorus 2.6-3.0 g/kg and 7.72-7.78 MJ/kg metabolizable energy.

***Key words**: biochemical composition, fodder value, green mass, hay, haylage, Onobrychis arenaria.*

INTRODUCTION

Forages are the major part of the diet of ruminant animals and provide energy, proteins and minerals. When feeding highly productive cows, the most common problem is meeting the protein need of animals. According to some scientists, due to the existing protein deficiency, agricultural enterprises lose about 30-35 % of their profits. This problem can be solved by increasing the production of high-protein fodder crops. Forage legumes are an essential component of agricultural systems in temperate regions of the world. Thanks to their nitrogen-fixing capabilities, they absorb nitrogen from the air, which reduces the need for nitrogen fertilizers.

Significant efforts to reduce the risk of bloat caused in ruminants by *Medicago* and *Trifolium* species have had limited success. One solution is to introduce other forage legume species that have the same beneficial qualities as alfalfa and clover, but without causing bloat. Forage legumes that contain moderate levels of secondary compounds such as condensed tannins and flavonoids offer some advantages to livestock nutrition. They increase nitrogen utilization efficiency within the digestive tract, reduce pasture bloat, provide resilience to resist parasites and reduce methane emissions into the environment from enteric fermentation (Mueller-Harvey et al., 2019).

Sainfoins (*Onobrychis* Mill.) are legume herbs, with 150 species distributed in many parts of the world, including West Asia, Europe, the western part of the United States and Canada. In comparison with other legumes species, the

Onobrychis species are less demanding to the soil and if there is enough moisture in the soil, it produces high yields even in the poorest soils, it is highly resistant to cold and drought, the fodder is characterized by high protein level and palatability, high condensed tannin content, provides beneficial effects to protect animals against bloat and increases protein absorption (Heckendorn et al., 2006; Hoste et al., 2014; Heuzé et al., 2020).

In Republic of Moldova the genus *Onobrychis* is represented by 4 species: *Onobrychis alba* (Waldst. & Kit.) Desv., *Onobrychis arenaria* Kit. D.C., *Onobrychis gracilis* Besser, *Onobrychis viciifolia* Scop.

Sand or Hungarian sainfoin, *Onobrychis arenaria* Kit. D.C. synonym *Onobrychis tanaitica* Spreng., *Onobrychis sibirica* (Besser) P.W. Ball is native to Eurasia and it has been cultivated in Ukraine since the beginning of the XX century. It has been used to create interspecies hybrids with common sainfoin, *Onobrychis viciifolia.* It is a perennial herb. Stems erect, branching, 40-90 cm high, with rare hairs or glabrous. Leaves pinnate with 6-15 pairs of elliptical or linear-lanceolate leaflets, 10-30 mm long, 2-5 mm wide. Racemes 5-9 cm long, multi-flowered. Florets purple-pink, 8-10 mm long. The pods are semi-pubescent, ovate, 5 mm long with short teeth on thorns and on the disk. The pods are flattened, indehiscent; each pod contains a single kidney-shaped seed, 4-6 mm in length. The plants bloom in May-June; seeds ripen in July. Due to its high drought resistance and nitrogen fixing ability, this species is suitable for fertilizing sandy, podzolic and calcareous soils, and for increasing the yields of subsequent crops on arable land.

The aim of this study was to evaluate some biological peculiarities, the biochemical composition and the fodder value of green mass, hay and haylage from sand sainfoin, *Onobrychis arenaria*.

MATERIALS AND METHODS

The local ecotype of sand sainfoin, *Onobrychis arenaria* that was cultivated in the experimental plot of the National Botanical Garden (Institute) Chişinău, N 46°58′25.7″ latitude and E 28°52′57.8″ longitude, served as subject of the research.

The green mass in the second growing year was harvested manually at 10 cm stubble height. The samples were harvested for the first time in early flowering period - in May, the second harvest was done on July 7, and the third harvest - on October 2. The green mass productivity was determined by weighing the yield obtained from a harvested area of 10 m^2. The leaves/stems ratio was determined by separating leaves and flowers from the stem, weighing them separately and establishing the ratios for these quantities, samples of 1.0 kg harvested plants were taken. For chemical analyses, the samples were dried at 65 ± 5ºC. The dry matter content was detected by drying samples up to constant weight at 105ºC. The prepared hay was dried directly in the field. The haylage was prepared from wilted green mass, shredded and compressed in well-sealed glass containers. After 45 days, the containers were opened, and the organoleptic assessment and biochemical composition of the haylage were determined in accordance with the Moldavian standard SM 108. The content of crude protein, crude fats, crude cellulose, calcium, phosphorus, soluble sugars, starch, ash, lactic, acetic and butyric acids was appreciated in accordance with standard laboratory procedures in Institute of Biotechnology in Animal Husbandry and Veterinary Medicine, Maximovca.

RESULTS AND DISCUSSIONS

Analyzing the agro-biological peculiarities of the local ecotype of sand sainfoin, *Onobrychis arenaria*, in the second growing season, it was established that the revival of plants was uniform, generative shoots developed in middle of April; they were characterized by moderate growth and development rates. At the end of May, the sand sainfoin plants reached 96 cm, the yield was 39.5 t/ha green mass or 6.56 t/ha dry matter, characterized by high content of leaves and flowers in the harvested mass (Table 1). Because of unfavorable meteorological conditions, high air temperatures and deficit moisture content of soil in May and early June, the revival of plants not was uniform. It was established that developed shoots grew about

60-68 cm, and the plants were cut for the second time, obtaining 19.23 t/ha green mass or 5.08 t/ha dry matter. The unfavorable meteorological conditions, the lack of rainfall and the very high air temperatures (38-41ºC) during the July-August affected the revival of sand sainfoin plants. A better growth and development rate was observed after the rain that fell at the middle of September, the formed shoots were erect and thicker, and reached 79 cm. The yield at the third harvest decreased in comparison with the previous harvests, and reached 10.03 t/ha green mass or 2.47 t/ha dry matter with optimal proportion of leaves (53%). The annual productivity of sand sainfoin, *Onobrychis arenaria*, in the second growing season, reached 68.76 t/ha green mass or 14.11 t/ha dry matter.

As a result of a research conducted by Matolinets & Voloshin (2016) in Perm region of Russia, it has been revealed that the three years' period average yield was 36.9 t/ha green mass or 7.53 t/ha of dry mass.

Table 1. Some biological peculiarities and the structure of the harvested mass depending on the harvest time of sand sainfoin, *Onobrychis arenaria*

Harvest time	Plant height, cm	Stem, g		Leaf + flower, g		Productivity, t/ha		Content of leaves and flowers in fodder, %
		green mass	dry matter	green mass	dry matter	green mass	dry matter	
First cut	96	6.28	0.88	9.00	1.62	39.50	6.56	64.8
Second cut	64	2.80	0.88	4.80	1.28	19.23	5.08	59.3
Third cut	79	3.72	1.27	5.31	1.43	10.03	2.47	53.0

Table 2. The biochemical composition and the fodder value of the green mass of sand sainfoin, *Onobrychis arenaria*

Indices	First cut	Second cut	Third cut
Crude protein, % DM	16.96	18.39	16.96
Crude fats, % DM	3.18	2.20	2.62
Crude cellulose, % DM	28.72	29.77	27.39
Nitrogen free extract, % DM	43.74	41.28	44.72
Soluble sugars, % DM	7.32	3.44	4.75
Starch, % DM	1.59	2.32	2.33
Ash, % DM	7.41	8.36	8.30
Nutritive units/ kg GM	0.17	0.20	0.26
Metabolizable energy, MJ/kg GM	1.73	2.47	2.53
Calcium, %	0.73	1.13	0.99
Phosphorus, %	0.25	0.25	0.27
Carotene, mg/ kg GM	45.45	43.45	42.00

The optimum use of forage resources in animal diets depends on the availability of detailed information on their chemical composition, biological properties and nutritional value, which may vary among plant species, cultivars, depending on age, growth stage and environmental conditions. The quality of the harvested green mass of sand sainfoin, *Onobrychis arenaria*, is presented in Table 2. It was found that in dry matter the crude protein varied from 169.6 to 183.9 g/kg, crude fats - from 22.0 to 31.8 g/kg, crude cellulose - from 273.9 to 297.7 g/kg, nitrogen free extract - from 412.8 to 447.2 g/kg, soluble sugars - from 34.4 to 73.2 g/kg, starch - from 15.9 to 23.3 g/kg, ash - from 74.1 to 83.6 g/kg, calcium - from 7.3 to 11.3 g/kg, phosphorus - from 2.5 to 2.7 g/kg. There was a significantly higher content of crude protein, crude cellulose and calcium in the green mass obtained after the second cut. The concentrations of crude fats and soluble sugars were high in the green mass at the first harvest and very low - in the green mass at the second harvest. The level of starch increased substantially in green mass at the second and third harvests.

Carotenoids, as part of the nutrients in the feed, support animal health and the quality of animal products. Animals cannot synthesize carotenoids. Plant carotenoids are precursors of vitamin A, together with vitamin E and polyphenols, which are natural antioxidants in ruminant diets. It was found that the concentrations of carotene decreased from 45.45 mg/kg fodder at the first haevers to 42.00 mg/kg fodder at the third harvest.

The content of dry matter, the concentration of nutrients and their digestibility influence the

feed and energy value of natural fodder. Therefore, 100 kg of green mass obtained at the first harvest contained 17 nutritive units and 173 MJ metabolizable energy, at the second harvest - 20 nutritive units and 247 MJ metabolizable energy and at the third harvest - 26 nutritive units and 253 MJ metabolizable energy for cattle. The estimated second season annual fodder productivity achieved 7600 nutritive units/ha, 1800 kg/ha digestible protein and 141.2 GJ/ha metabolizable energy.

Several literature sources describe the biochemical composition and nutritional performance of sainfoin whole plants. Gryazeva (2005), reported that *Onobrychis arenaria* green mass contained 238.4- 244.6 g/kg dry matter with 18.57-19.31% crude protein, 2.29-2.36% crude fats, 29.18-29.41% crude cellulose, 41.93-44.02% nitrogen free extract, 5.95-6.90% ash, but *Medicago sativa* green mass contained 248.9-269.6 g/kg dry matter with 18.62-20.66% crude protein, 2.49-2.52% crude fats, 31.18-32.60% crude cellulose, 37.20-39.17% nitrogen free extract, 7.02-9.09% ash. According to Pankov (2013), *Onobrychis arenaria* green mass harvest in flowering period contained 18.4% crude protein, 3.1% crude fats, 27.8% crude cellulose, 41.9% nitrogen free extract, 8.8% ash, 11.7 g/kg calcium and 1.7 g/kg phosphorus. Voloshin et al. (2015), found that the concentrations of nutrients and energy in the dry matter of tested cultivars of *Onobrychis arenaria*, at the first harvest, were 14.51-17.70% crude protein, 2.47-2.72% crude fats, 27.13-28.82% crude cellulose, 6.13-6.79% minerals, 6.09-6.44% sugars 92.25-137.11 mg/% carotene, 0.78-0.83 nutritive units/kg, 9.81-10.12 MJ/kg metabolizable energy and 144 g digestible protein per nutritive unit, but in the green mass at the second harvest, respectively, 15.42-15.92% crude protein, 2.45-2.60% crude fats, 21.24-24.38% crude cellulose, 3.00-4.20% minerals, 5.34-5.61% sugars, 142.90-152.43 mg/% carotene, 0.92-1.01 nutritive unit/kg, 10.61-11.17 MJ/kg metabolizable energy. Demydas et al. (2019) compared the forage quality of green mass from different species of sainfoin and found that the chemical composition of *Onobrychis arenaria* was 20.5-20.6% crude protein, 4.16-4.22% crude fats, 21.5-21.9% crude cellulose, 8.09-8.15% ash, 46.00% nitrogen free extract, 13.2-13.3 g/kg calcium and 6.2-6.5 g/kg phosphorus; *Onobrychis viciifolia* contained, respectively, 19.3-19.4% crude protein, 3.48-3.62% crude fats, 21.2-21.6% crude cellulose, 7.80-7.98% ash, 48.00% nitrogen free extract, 13.4-13.5 g/kg calcium and 5.2-5.6 g/kg phosphorus; *Onobrychis transcaucasica* - 20.1-20.3% crude protein, 4.07-4.20% crude fats, 21.5-21.6% crude cellulose, 8.06-8.16% ash, 46.00% nitrogen free extract, 12.6- 13.3 g/kg calcium and 6.4-6.6 g/kg phosphorus.

The use of forage conservation methods to supply roughage to herbivores at critical times of production is an excellent strategy in animal production. Giving that the main ones are hay, silage and haylage, they require peculiar characteristics to be conserved. The nutrition provided by hay is vital to keep the animal healthy and to protect their digestive health. The biochemical composition and fodder value of the hay prepared from sand sainfoin, *Onobrychis arenaria,* is presented in Table 3. The dry matter of prepared hay contained 163.8-167.9 g/kg crude protein, 16.5-17.0 g/kg crude fats, 315.2-366.7 g/kg crude cellulose, 377.1-413.7 g/kg nitrogen free extract, 75.5-83.6 g/kg ash, 7.7-9.9 g/kg calcium and 2.3-2.9 g/kg phosphorus. It is known that the digestibility of nutrients in hay is lower. Therefore, the nutritive value of 100 kg of hay from sand sainfoin was 46-62 nutritive units and 786-866 MJ metabolizable energy.

Table 3. The biochemical composition and the fodder value of the hay from sand sainfoin, *Onobrychis arenaria*

Indices	First cut	Second cut
Crude protein, % DM	16.38	16.79
Crude fats, % DM	1.70	1.65
Crude cellulose, % DM	36.67	31.52
Nitrogen free extract, % DM	37.71	41.37
Soluble sugars, % DM	3.36	2.10
Starch, % DM	1.24	1.39
Ash, % DM	7.55	8.36
Nutritive units/kg DM	0.46	0.62
Metabolizable energy, MJ/kg DM	7.86	8.66
Calcium, % DM	0.77	0.99
Phosphorus, % DM	0.23	0.29

Some authors mentioned various findings about the quality of the hay from *Onobrychis* species. Medvedev & Smetannikova (1981) remarked that sand sainfoin hay contained: 11.2-11.8% digestible protein, 1.8-2.9% crude fats, 5.6-

6.1% ash, 19.0-27.7% crude cellulose, 32.9-43.8% nitrogen free extract and 0.58 nutritive units/kg. Ryabinina (1998) reported that *Onobrychis arenaria* hay contained: 17.7% crude protein, 4.45% ash, 22.8% crude cellulose, 45.6% nitrogen free extract, 0.79% calcium, 0.21% phosphorus, 0.61 nutritive units/kg and 10.1 MJ/kg metabolizable energy. Dzyubenko & Abdushaeva (2012) reported that *Onobrychis arenaria* hay contained: 15.4% crude protein, 3.2% crude fats, 6.2% ash, 24.9% crude cellulose, 34.0% nitrogen free extract and 0.54 nutritive units/ kg.

It is advisable to feed lactating cows, in winter, with haylage, because its properties are close to those of the green mass of grasses, and the nutritional value of dry matter - to grass flour. As for the organoleptic properties, the haylages prepared from *Onobrychis arenaria* consists of green-olive leaves and yellowish-green stems; has a pleasant smell of pickled vegetables; the texture of the plants stored as haylage was preserved well, without mold and mucus. The quality of sand sainfoin haylage prepared from green mass obtained at the second and third cuts is shown in Table 3. It has been determined that the pH index was 4.75-5.16, the concentrations of organic acids reached 60.5-62.8 g/kg, and most amounts of organic acids were in fixed form. The butyric acid was detected in fixed form - 0.3-0.4 g/kg.

Table 4. The quality of the haylage from sand sainfoin, *Onobrychis arenaria*

Indices	Second cut	Third cut
pH index	5.16	4.75
Content of organic acids, g/kg	60.5	62.8
Free acetic acid, g/kg	0.6	1.7
Free butyric acid, g/kg	0	0
Free lactic acid, g/kg	4.1	4.7
Fixed acetic acid, g/kg	2.4	4.6
Fixed butyric acid, g/kg	0.4	0.3
Fixed lactic acid, g/kg	53.0	51.5
Total acetic acid, g/kg	3.0	6.3
Total butyric acid, g/kg	0.4	0.3
Total lactic acid, g/kg	57.1	56.2
Acetic acid, % of organic acids	4.96	10.03
Butyric acid, % of organic acids	0.66	0.48
Lactic acid, % of organic acids	94.38	89.49
Crude protein, % DM	16.49	17.49
Crude fats, % DM	2.61	2.74
Crude cellulose, % DM	30.03	27.82
Nitrogen free extract, % DM	42.89	43.93
Soluble sugars, % DM	2.58	1.46
Starch, % DM	1.67	2.07
Ash, % DM	7.99	7.71
Metabolizable energy, MJ/kg DM	7.72	7.78
Calcium, % DM	0.99	0.93
Phosphorus, % DM	0.30	0.26

The haylage prepared from green mass obtained at the second harvest was characterized by optimal content of lactic acid and low content of acetic acid, in comparison with the haylage prepared from the green mass obtained at the third harvest. The dry matter in haylages prepared from sand sainfoin, contained 16.49-17.49% crude protein, 2.61-2.74% crude fats, 27.82-30.03% crude cellulose, 42.89-43.93% nitrogen free extract, 1.46-2.58% soluble sugars and 1.67-2.07% starch, 7.71-7.99% ash, 0.93-0.99% calcium and 0.26-0.30% phosphorus, 7.72-7.78 MJ/kg metabolizable energy. There was a significantly higher content of crude protein, nitrogen free extract and starch in the haylage obtained from green mass after the third cut.

Shitov (2008) found that pure haylage from sainfoin was characterized by pH 4.6, concentrations of lactic acid 1.31%, acetic acid 0.81%, butyric acid 0.24%, but sainfoin haylage conserved with *Lactobacillus* - pH 4.2, lactic acid 1.25%, acetic acid 0.47% and butyric acid 0 %. Morozkov & Maysak (2020) reported that *Onobrychis arenaria* haylage contained: 16.96% crude protein, 2.59% crude fats, 26.15% crude cellulose, 4.86% sugars, 10.63 g/kg calcium, 2.79 g/kg phosphorus, 22.90 mg/kg carotene, 9.50 MJ/kg metabolizable energy. Sainfoin haylage had positive effect on immuno-biochemical parameters of blood of cows and their reproductive functions.

CONCLUSIONS

The local ecotype of sand sainfoin, *Onobrychis arenaria*, in the second growing season, was characterized by optimal growth rate and regenerative capacity after mowing, making it possible to cut it three times per season, reaching a productivity of 68.76 t/ha green mass or 14.11 t/ha dry matter.

The biochemical composition of the green mass varied depending on the harvest time: crude protein 169.6-183.9 g/kg, crude fats 22.0-31.8 g/kg, crude cellulose 273.9 - 297.7 g/kg, nitrogen free extract 412.8 - 447.2 g/kg, soluble sugars 34.4-73.2 g/kg, starch 15.9-23.3 g/kg, ash 74.1-83.6 g/kg, calcium 7.3-11.3 g/kg, phosphorus 2.5-2.7 g/kg, carotene 42.00-45.45 mg/kg.

The estimated fodder productivity achieved 7600 nutritive units/ha, 1800 kg/ha digestible protein and 141.2 GJ/ha metabolizable energy.
The hay prepared after the first and second harvests contained 163.8-167.9 g/kg crude protein, 16.5-17.0 g/kg crude fats, 315.2-366.7 g/kg crude cellulose, 377.1-413.7 g/kg nitrogen free extract, 75.5-83.6 g/kg ash, 7.7-9.9 g/kg calcium and 2.3-2.9 g/kg phosphorus, 7.86-8.66 MJ/kg metabolizable energy.
The biochemical and the fodder value of the prepared haylages were: pH 4.75-5.16, lactic acid 51.5-53.0 g/kg, acetic acid 2.4-4.6 g/kg, butyric acid 0.3-0.4 g/kg, crude protein 164.9-174.9 g/kg, crude fats 26.1-27.4 g/kg, crude cellulose 278.2-300.3 g/kg, nitrogen free extract 428.9-439.3 g/kg, sugars 14.6-25.8 g/kg, starch 16.7-20.7 g/kg, calcium 9.3-9.9 g/kg, phosphorus 2.6-3.0 g/kg and 7.72-7.78 MJ/kg metabolizable energy.
The green mass, hay and haylage obtained from sand sainfoin, *Onobrychis arenaria*, contain a lot of nutrients, which make them suitable to be used as a part of diverse livestock diets.

ACKNOWLEDGEMENTS

The study has been carried out in the framework of the projects: 20.80009.5107.02 *"Mobilization of plant genetic resources, plant breeding and use as forage, melliferous and energy crops in bioeconomy"* and 20.80009.5107.12 *"Strengthening the «food-animal-production» chain by using new feed resources, innovative sanitation methods and schemes"*

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

THE EFFECT OF USE OF MIXED RED GINGER (*ZINGIBER OFFICINALE* VAR. *RUBRUM*) AND TURMERIC (*CURCUMA LONGA*) IN THE RATION ON PERFORMANCE AND CARCASS QUALITY OF BROILER

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Abstract

The aim of this study was to find out the best dose of red ginger (Zingiber officinale var. rubrum) and turmeric (Curcuma longa) mixture with a ratio of 1:1 which could produce the best performance and carcass quality of broiler. The experiment used 100 broiler day old chicken with a Completely Randomized Design (CRD). There were four kind of treatments (R_0: Based ration, R_1: Based ration +0.25% red ginger + 0.25% turmeric, R_2: Based ration + 0.5% red ginger + 0.5% turmeric, R_3: Based ration +0.75% red ginger + 0.75% turmeric, where each treatment was repeated five times and each repeated consist five broiler chicks. Analysed variables were feed consumption, body gain weight, feed conversion, carcass weight, fat abdominal and meat cholesterol. Statistical analysis indicated that addition of mixed Red Ginger and turmeric meal did not significantly affect (P>0.05) on feed consumption, abdominal fat and significantly to gain on body weight, feed conversion, carcass weight and meat cholesterol. It can be concluded that by using until 1% in the ration produced good performance and carcass quality of broiler.

Keys words: *broiler, carcass quality, performance, Red Ginger, turmeric.*

INTRODUCTION

Broilers are meat-producing poultry that have considerable potential in meeting people's needs for animal protein. The existence of quality chicken meat commodities requires the requirement to be free from drug residues. In order to meet consumer demands in the form of quality chicken meat, it is usually done by manipulating the nutritional content or the source of certain ingredients in the ration. One of the efforts that can be done is providing feed additives.

Feed additives are ingredients that are deliberately mixed into animal feed with the aim of increasing productivity, health, and the nutritional condition of livestock and not to meet the nutritional needs of livestock (Sinurat et al., 2009) The feed additives that are currently widely used are herbal raw materials, in this study red ginger (*Zingiber officinale* var. *rubrum*) and turmeric (*Curcuma longa*) are used. Red ginger contains bioactive components in the form of oleoresin and gingerol essential which functions to help optimize organ function (Arifin et al., 2013). Essential oils help digestive enzymes work so that the feed rate increases and along with the growth rate, meat production will increase. Ginger has the power to increase appetite, strengthen the stomach and improve digestion. The essential oils released by the ginger rhizome are illuminated by the mucous membranes of the large stomach and intestines, which results in an empty stomach and the livestock will consume feed (Setyanto et al., 2012) The nature of gingerol as an anticoagulant, which is to prevent blood clots, is also thought to be able to reduce cholesterol levels. Red ginger essential oil is believed to have properties to inhibit the growth of microorganisms (Rahmawati, 2013). Ginger in the ration of broilers can significantly reduce abdominal fat compared to control feeding (Latief et al., 1997).

Turmeric is a type of plant that can be used to replace synthetic antibiotics, because it contains active or bioactive compounds that have functions such as chemicals in synthetic antibiotics. The active compounds are curcumin and essential oils. The essential oil content in turmeric is 3-5% and curcumin 2.5-

6% (Rukmana, 2005). Curcumin compounds and essential oils contained in turmeric rhizomes are thought to increase production levels and facilitate the excretion of bile in broilers, resulting in decreased meat cholesterol content (Legowo, 2004). The addition of saffron meal to the feed indirectly affects feed consumption and absorption of food substances so that it can form meat production and the percentage of meat carcass will increase (Mide, 2012). The curcumin content of turmeric has an antibacterial and antioxidant function. Curcumin contained in turmeric has properties that can affect appetite because it can accelerate the emptying of stomach contents so that appetite increases and expedites bile excretion thereby increasing the activity of the digestive tract (Purwanti, 2008). The curcumin content can reduce the percentage of abdominal fat in broiler meat. The use of phyto-pharmacy in the form of red ginger and turmeric can increase the final weight so that it can increase the slaughter weight and carcass weight of broiler chickens. The curcumin content in turmeric can also reduce abdominal fat and the addition of ginger meal can also reduce abdominal fat.

The addition of red chilies and black pepper as feed additives with a ratio of 0.5% red chili and 0.5% black pepper can increase body weight, feed intake, feed conversion and can reduce abdominal fat (Puvaca et al., 2014). Feed additives at a level of 1% increase the performance and overall quality of broiler chickens where the addition of feed additives is given, namely red pepper with a level of 0.5% and black pepper with a level of 0.5% (Safa, 2014). Because of the effect of essential oils on the function or work of the digestive tract, especially the small intestine, and are toxic in excessive doses, the use of the right mixed red ginger and turmeric meal is expected to increase the body's metabolism and metabolism that affects the digestive tract cells. Therefore this research was conducted to study the effect of adding mixed red ginger and tumeric meal in the ration on the performance and carcass quality of broiler chickens.

MATERIALS AND METHODS

The study used 100 DOC broiler chicken with the average body weight of 42.34 gram (coefficient of variation 0.23%). DOC broiler were kept in deep litter system until the age of 35 day, 20 pens were used, sized 90 cm x 90 cm x 60 cm (length x width x height). Each pen consisted of 5 chickens.

The feed additives used are red ginger and turmeric mixed in the feed in the form of meal. The dose given in each treatment is different with the ratio between red ginger and turmeric, which is 1:1.

The feed ingredients used for the ration consist of yellow corn, soybean meal, fish meal, fish oil, salt, top mix, methionine and bone meal. The preparation of a ration for broiler chickens contains 22% protein and a metabolic energy of 3000 kcal/kg (Daghir, 1998). The composition of experimental rations is in Table 1 and the nutrient and metabolism energy content of basal ration are in Table 2.

The variables measured were feed consumption, weight gain, feed conversion, carcass weight, abdominal fat weight and meat cholesterol. Statistical test was performed by analysis of variance and differences between treatments effects were examined using Duncan's multiple range test (Daghir, 1998)

Table 1. Basal diets composition

Ingredients	Amount
	%
Yellow corn meal	56.0
Meat Bone Meal	4.5
Soybean Meal	22
Fish Meal	3
Salt	0.2
Corn Glutean Meal	12.5
Lysine	0.2
Methionine	0.1
Bone meal	1.0
Top Mix	0.5
Total	100

Source: Calculations using trial and error Microsoft Excel (2019).

Table 2. Nutrient and Metabolic Energy Content of Basal Ration

Ingredient	Amount
ME (kcal/kg)	300.08
Crude Protein (%)	22.42
Crude Fat (%)	3.61
Crude Fiber (%)	3.95
Calcium (%)	1.00
Phosphorus (%)	0.52
Lysine (%)	1.30
Methionine (%)	0.64
Cystine (%)	0.42
Triptophane (%)	0.26

RESULTS AND DISCUSSIONS

The results of addition of mixed red ginger and turmeric rations of broiler chickens on the feed consumption, body weight gain, feed conversion, carcass weight, abdominal fat and meat cholesterol for each treatment can be seen in Table 3.

Effect of treatment on feed consumption

The results of the analysis of variance, it showed that the giving of mixed red ginger and turmeric in broiler rations had a significant effect ($P<0.05$) on feed intake. Rations containing mixed red ginger and turmeric produce a fragrant aroma containing active substances, namely curcumin and essential oils which can increase appetite.

Table 3. Average feed consumption, weight gain, feed conversion, carcass weight, abdominal fat weight and cholesterol content of broiler chicken

Variable	P0	P1	P2	P3
Feed Intake (g)	2803.90[a]	2537.60[b]	2814.00[b]	2420.19[a]
Body gain (g)	1428.60[a]	1505.70[b]	1555.7[b]	1401.32[a]
FCR	1.96[a]	1.69[b]	1.56[b]	1.83[a]
Carcass weight (g)	924.20[a]	1129.80[b]	1186.60[b]	900.20[a]
Abdominal fat (g)	13.68[a]	12.40[a]	12.45[a]	11.92[a]
Meat cholesterol (mg/100 g)	91.50[a]	80.55[b]	80.33[b]	78.55[b]

Note: different superscript shows significant differences.

The active ingredients of curcumin in mixed red ginger and turmeric have cholagogic activity, which functions to increase the production and secretion of bile which is useful for emulsifying fat and can reduce body fat levels. Meanwhile, essential oils can stimulate an increase in the relaxation of the small intestine so that there will be an increase in digestion and absorption of feed substances. The addition of red ginger and turmeric with a dose of P3 (1.5%) will decrease feed consumption due to the presence of active substances in the form of essential oils which cause a pungent odor, added with the bitter taste of turmeric. In accordance with the opinion of Swastike (2012) that the palatability of the ration will decrease in the presence of a bitter taste and pungent odor, from turmeric so that giving turmeric as much as 4% can reduce ration consumption significantly.

Effect of Treatment on Weight Gain

From Table 3, it can be seen that the average body weight gain in treatment R1 and R2 was significantly higher ($P<0.05$) compared to treatment R0 and R3. When associated with ration consumption, this means the level of mixed red ginger and turmeric is 0.5-1.0% in the ration can increase body weight gain, while at the level of 1.5% there is a decrease in body weight gain. This is the effect of essential oils on the work of the digestive tract, especially the small intestine, and is toxic at excessive doses. In R3 treatment, it is seen that there are limitations in the function or work of essential oils so that there is a decrease in body weight gain, even though it is still within normal limits. The content of the active ingredient components, the beneficial value is the function of essential oils because the curcumin content is undetectable. The relationship between essential oils and body weight produced shows a working effect on protein digestibility in the formation of animal body tissues, including meat. The facts found provide an illustration that the mixture of red ginger and turmeric flour does not have a negative effect on average body weight gain, so that it can become a feed additive in broiler chicken rations, especially at the right dose. Afifah and Lentera (2002) state that adding ginger can increase body weight and reduce blood cholesterol levels in broiler chickens due to the work of curcumin and essential oils from turmeric.

Effect of Treatment on Feed Conversion

In Table 3, it can be seen that the addition of a mixture of red ginger and turmeric meal to the conversion value gives a positive increase. The average value of feed conversion for the addition of 0.5-1.0% mixture of red ginger and turmeric meal was significantly lower than the basal ration and rations containing 1.5% mixture of red ginger and turmeric. The ration conversion value is influenced by feed consumption and body weight gain. The results showed that body weight gain tended to increase in line with the increasing use of a mixture of red ginger and turmeric meal in the ration, while the consumption of treatment

rations was not different, causing the ration conversion value to tend to decrease. The presence of essential oils in red ginger can help digestion by stimulating the nervous system of secretions, so that gastric juice which contains enzymes such as lipase, amylase and trypsin, is secreted into the stomach and intestines, as a result the chicken is able to remodel all complex amylose, so that it is easily absorbed and broken down into meat. In line with the opinion of Desmayati (2007) which state that the bioactive substances contained in herbal ingredients such as turmeric and red ginger are thought to contain substances that can improve carbohydrate metabolism and metabolize fat in the body, thereby increasing feed efficiency and livestock health.

Effect of Treatment on Carcass Weight

Based on the Duncan test, the carcass weight of broiler chickens for treatment P1 (0.5%) and P2 (1.0%) gave a significantly higher effect than treatment P0 (0.0%) and P3 (1.5%). The addition of red ginger and turmeric can increase body weight in broiler chickens. This is in accordance with the statement of Barton and Hart (2001). Addition of antibiotic feed functions to reduce the number of pathogenic microbes in the digestive tract of chickens, so that it can increase the body weight of chickens by about 3.9% and increase the efficiency of feed use by around 2.9%. Carcass weight is closely related to the live weight of chickens at harvest time. In addition, part of the ration that is very influential for carcass formation is the protein content of the ration (Setiadi et al., 2011). The addition of red ginger and turmeric can increase the percentage of carcass weight because red ginger contains essential oils. According to Setyanto et al. (2012), essential oils can stimulate the mucous membranes in the large stomach and intestines which can cause the stomach to become empty and livestock will consume food and if the ration is added with turmeric, it will indirectly affect feed consumption and absorption of feed substances that will be form the meat and the percentage of meat carcass will be optimal (Mide, 2012).

Effect of Treatment on Abdominal Fat

Abdominal fat is fat found around the gizzard, abdominal muscles and small intestine (Akhadiarto, 2010). The decreasing of the ration consumption, the nutrients absorbed also decreased, including fat as well as energy, with the decrease in energy, the fat in chickens that occurred was also low, seen in the decreased abdominal fat. The presence of phytochemicals found in ginger meal can bind fat or inhibit cholesterol formation (Argawal and Rao, 2000). Bioactive substances such as essential oils and curcumin, which play a role in improving the work of the digestive organs, stimulate the bile walls to release bile and stimulate the release of pancreatic juice which contains the enzyme lipase to improve fat digestion (Agustina, 2006). Furthermore, Supomo et al. (2016) stated that the content of essential oils stimulates the release of pancreatic juice, where the pancreatic juice releases lipase enzymes which can break down glycerol fatty acids so that the fat formed is reduced. The chemical compounds in turmeric in herbal ingredients can reduce fat in the body, play a role in the process of bile and pancreatic secretion that is excreted through feces (Rahayu and Budiman, 2005). The reduction in abdominal fat weight did not have a significant effect because the broiler chickens were still in their infancy. The age factor of the chicken is one of the factors causing the accumulation of abdominal fat in the body of the chicken. In chicken livestock, fat tissue begins to form rapidly at the age of 6-7 weeks, then from that time the accumulation of fat continues to accelerate, especially abdominal fat at the age of eight weeks so that chicken body weight increases rapidly (Pratikno, 2011).

Meat Cholesterol

Analysis of variance showed that by addition of mixes red ginger and turmeric has significantly effect ($P<0.05$) on the meat cholesterol broiler chicken. The result indicated that by treatment adding until 1% gave the best results of meat cholesterol. Red ginger and turmeric produces antioxidants such as: scopoletin, nitric oxide, vitamin C and vitamin A, and has the efficacy to increase the secretion of bile and substance NO (Nitrit Oxide) that can stimulate the excretion of cholesterol through feces. Flavonoid in red ginger is one of the phyto-chemical groups that have the same structure, namely polyphenols, whose mechanism can

reduce cholesterol levels due to HMG-CoA (HydroxyMethyl Glutatyil-CoA) reductase activity, reduce the activity of the enzyme acyl-CoA cholesterol acyltransferase (ACAT), and reduce cholesterol absorption in the digestive tract of proteins for hormones, one of which is the hormone insulin that can increase the number of LDL receptors (low density lipoprotein) hepatic and extra hepatic.

CONCLUSIONS

The present study shows the potential of Mixture red ginger and turmeric basal ration significantly affected on performance broiler chicken and can be natural antibiotics from herbal

The addition of mixture red ginger and turmeric up to 1% in the ration could have positive impact on the growth of broiler chickens and carcass quality so as to produce healthy chicken meat, low in cholesterol, so that it is safe for consumption.

ACKNOWLEDGEMENTS

I would like to thank to Dani Garnida and M.Panji Ismail who helped write this paper.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

INFLUENCE OF MILK SUBSTITUTES AND GROWTH ACCELERATOR ON PERFORMANCE AND HEALTH IN WEANING PIGS

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Abstract

A scientific experiment was conducted in Agricultural Institute - Shumen with pigs, divided into three groups. Each group contained three litters, equalized by number of piglets. All animals were fed at will and received the Neopigg Rescuemilk and Neopigg Smooth supplements in I group, Neopigg Rescuemilk + Neopigg Smooth + growth accelerator "AXCLERA-P" (200 g per pig for the entire period up until weaning) - II group, Neopigg Smooth + AXCLERA-P (200 g per pig for the entire period up until weaning) - III group. Throughout the entire experiment, the consumption was reported daily, as well as health and percentage of dropout animals, live weight at birth, at weaning on the 28th day and in the end of the experiment at the 49th day. The addition of Neopigs Rescuemilk, Neopigs Smooth and Axclera-P day had a significant positive effect on average daily gain and weaning weight. When including dairy supplements during the suckling period (0-28th day), live weight at birth should also be taken into account, which is also a significant factor in the animals' development.

Key words*: accelerator, health, milk substitute, pigs, suckling pigs.*

INTRODUCTION

Contemporary pig breeding is implausible without the use of various growth stimulants, especially in young animals with an underdeveloped digestive and immune systems. In recent years, while assessing the urgency of the problem, scientists around the world have turned to the search of new natural and environmental growth stimulants, replacements for the nutritional antibiotics used today. Numerous feed additives are available on the market to help boost pigs' immune system, regulate the gut micro biota and reduce the negative effects of weaning and other environmental challenges (Yanhong et al., 2018)

A requirement of modern pig breeding is to know the precise nutritional needs of pigs and their breeding to develop adapted nutrition strategies and thus increase their productivity (Brossard et al., 2017).

Commonly used feed additives include acidifying agents, zinc and copper, prebiotics, directly fed germs, yeast, nucleotides and plant extracts that lead to improved pig breeding efficiency or enhanced animal immune function (Yanhong et al., 2018).

Organic acids are also widely used as nutritional supplements. They can cause a decrease in pH in the gastrointestinal tract and thus counteract the germs. Therefore, they are considered to be an alternative to the growth-enhancing antibiotics used for pigs. In addition, they play a role in reducing the ratio of harmful germs in the gastrointestinal tract (Kwak et al., 2017).

Nutritional strategies to improve feed efficiency are of particular interest as their use reduces environmental impact and improves the profitability of pig production. The pig digestive system lacks specific enzymes that break down some of the chemical bonds present in the non-starch polysaccharide fraction of the ration (i.e., arabinoxylans and β-glucans). Supplements are therefore used strategically to improve nutrient absorption and increase growth (Torres- Pitarch et al., 2020).

Energy is one of the most expensive nutrients in feeds. Since lipids are a concentrated source of energy, their incorporation has been known to affect growth rate and nutrition efficiency, but also known to affect rationing, food dustiness and granule quality (Kerr et al., 2015). On a global scale, feed has been reported to account for about 70-80% of the

cost of producing animals. That necessitates the creation of alternative nutrients that are inexpensive and at the same time able to supply the nutrients needed by animals for optimal growth and growth productivity (Achadu et al., 2018).

Such is the supplement AXCELERA-P, which is a complete feed for piglets before weaning. The composition also includes whey powder, whole soybean, oatmeal, palm oil, potato protein, brewer's yeast, wheat, monodicalcium phosphate, magnesium oxide.

Whey contains 20% of total milk protein. It has high biological value with abundant amino acids. Whey proteins consist mainly of α-lactoalbumin and β-lactoglobulin, which have positive health effects. Whey protein supplements improve protein synthesis, mineral absorption and circulation. It has various functional characteristics such as antioxidant capacity and thermal stability (Kim et al., 2016).

Soybean meal (SBM) and other soy based products contribute for the high quality protein in pig rations, because soybean protein is rich in the following limited amino acids: lysine, threonine and tryptophan which have low concentration levels in the most used cereals. Pigs digest amino acids in soybean protein in much higher levels, rather than those in other sources of protein (Stein et al., 2013).

Oat is a grain, rich in fibre, fat (2-12%) and has almost double the amount of lysine, compared to corn, and is rich in Vitamin B1, B2, B6 and Vitamin A, K and E. Oat helps maintain a normal gut function, reduces the risk of constipation and diarrhea and minimizes stressful behavior (Christy, 2018).

An advantage of using palm oil as an energy resource is the high caloric density and lack of fibre (Durán, 1994).

Potato protein concentrate is considered a valuable source of essential amino acids that can replace animal protein in pig production. Potato fibre preparations have been studied as a potential source of functional dietary fibre for feed and food. Dietary proteins and fibre have a great influence on the functional status of the gastrointestinal tract and, accordingly, on the immunology, health and productivity of pigs (Tusnio et al., 2011).

Brewing yeast is an agro-industrial by-product of brewing beer. It is valuable for animal husbandry because of its high protein content (about 45% of dry matter), which is high in amino acids, particularly lysine, vitamins, carbohydrates and fats (Achadu et al., 2018).

Over 40% of produced wheat and barley is used in livestock production. They are the main sources of energy in the ration for breeding and fattening pigs.

Compared to corn, wheat has significantly higher protein content - 12-12.5%. It is rich in starch and energy, but contains small amounts of fats (approximately 2%) and fibres (2-3%). The digestibility is high, superior to barley and oats in this respect, but not to corn (Ball and Magowan, 2012).

The aim of the experiment was to establish the effect of the addition of “Neopigg Rescuemilk” and “Neopigg Smooth” milk substitutes and growth accelerator “AXCLERA-P” on the growth, health and the percentage of dropping out in pigs.

MATERIALS AND METHODS

A scientific experiment was conducted in Agricultural Institute - Shumen with pigs, divided into three groups. Each group contained three litters, levelled by number of piglets. All animals were fed at will and received not only breast milk, but also “Neopigg Rescuemilk” and “Neopigg Smooth” milk substitutes and growth accelerator “AXCLERA-P”, according to the experiment scheme (Table 1).

Table 1. Experiment scheme

Groups	I group	II group	III group
Number of animals	37	36	39
Supplement	Neopigg Rescuemilk +	Neopigg Rescuemilk +	Neopigg Smooth +
	Neopigg Smooth	Neopigg Smooth +	AXCLERA-P (200g per pig for the entire period until weaning)
		AXCLERA-P (200g per pig for the entire period until weaning)	

Throughout the entire experiment, the consumption of milk substitutes was reported daily, as well as health and percentage of dropout animals. Live weight at birth, at weaning on the 28th day and in the end of the

experiment at the 49th day were the other controlled traits.

RESULTS AND DISCUSSIONS

The results characterizing the reproductive traits and analysis of the F-test variance are presented in Table 2. The analysis of the results shows that the animals in the study groups were of high live weight at farrowing (1.6 kg) and normal development during the suckling period. The variation of signs was in the low range, with the influence of the trait "group" being reliable for all signs related to the weight dimensions and for the increase from 28 to 49 days (P≤0.05). A low degree of significance was observed for the other traits characterizing the 49th day growth.

Determination coefficients are medium to high in value for most of the reproductive traits (R2 = 62-82%) and show that the study factor accurately reflects the variation in traits in the model of analysis used. Low values were found for the increase from farrowing to the 49th day.

Table 2. Reproductive ability, determination coefficient (R^2) and ANOVA F-test

Traits	No.	Live weight, kg				Average daily gain, g			
		0	21th	28th	49th	0-21	0-28	28-49	0-49
	n=83	At weaning	21th	28th	49th	0-21	0-28	28-49	0-49
$\bar{x}$		1.63	5.39	8.90	13.34	198.25	269.27	243.96	283.87
SD		0.37	1.06	1.59	2.10	96.46	55.05	42.19	63.32
R^2		0.67	0.69	0.78	0.82	0.053	0.055	0.062	0.034
Groups	3	+	+	+	+	n.s.	n.s.	+	n.s.

Significance of differences: * - P≤0.05; n.s. - no significance.

The development results of piglets with the inclusion of various supplements during the suckling and post-weaning periods, expressed in terms of average daily gain and live weight are presented in Table 3.

The highest average daily gain for the period up to the 21st day was in pigs from II group, compared to those from group I (by 21.88%, p≤ 0.05) and group III (by 28.64%, p ≤ 0.01). The higher gain was probably due to the higher live weight at birth of the piglets (1.75 kg in II vs. 1.62 kg in I and 1.52 kg in III) in this group. Regarding the average daily gain on 28th day (at weaning), proven differences between the three experimental groups were reported. The second group with added milk N. Rescuemilk, N. Smooth and Axclera-P had a higher gain of 6.04% (P≤0.05) than the first group and 12.94% (P≤0.05)) compared to those in the third group.

The addition of the three types of milk had a positive effect on live weight at weaning. The live weight of the weaned piglets was highest in group II (9.41 kg), followed by those in group I (8.83 kg) and the lowest live weight was group III (8.30 kg), differences between the second group and the other two experimental groups were demonstrated at P≤0.05-P≤0.001. The trend of development during the period from the 28th to the 49th day remained the same as during the suckling period. Animals in the second group had a higher gain (0.257 kg) and live weight (14.08 kg) compared to those in Group I and III, with differences in both indicators being demonstrated at P≤0.01-P≤ 0.001.

Valuable nutrients are given by milk products when added for breastfeeding pigs. In addition, these nutrients were easily absorbed by the young animal's immature gastrointestinal system. The macronutrients of milk are butterfat, casein and whey proteins and lactose. Also, micronutrients such as minerals (e.g. calcium, phosphate), vitamins (e.g. vitamin A), immunoglobulins and enzymes determine its nutritional value. Nukamel's research reported by Croes (2014) showed that the digestible milk supplement (Nukamix) contained milk and vegetable protein (coconut and milk fats), and in the suckling and post-weaning period, better results were established for gain and feed intake. Considering the positive impact of added dairy products during the suckling period on the development and conservation of piglets, we should also note the significant effect on live weight at birth found not only in

our study but in studies by numerous authors. Taking into account the greater farm impact, Pustal et al. (2015) found no effect of including additional milk in suckling pigs. Results from the study by Wolter et al. (2002) indicated that birth weight of piglets has a significantly greater effect on post-weaning gain rather than the increase of nutrient intake by supplemental dairy intake.

Table 3. Average daily gain and live weight

			Live weight, kg				Average daily gain, g				Live weight, kg				Average daily gain, g			
Factors		n	0	21th	28th	49th	0-21	0-28	28-49	0-49	0	21th	28th	49th	0 -21	0 -28	28-49	0-49
LSC		83	1.62 ± 0.40	5.36 ± 0.12	8.85 ± 0.17	13.27 ± 0.23	195.96 ± 10.56	267.51 ± 6.02	242.73 ± 4.59	282.60 ± 6.70								
G	1	30	1.60 ± 0.19	5.32 ± 0.19	8.83 ± 0.28	13.10 ± 0.37	185.67 ± 17.36	267.57 ± 9.89	239.57 ± 7.55	277.97 ± 11.51	**1-2 ***	**1-2 ***	**1-2 ***	**1-2 ****	**1-2 ***		**1-2 ***	
	2	31	1.75 ± 0.65	5.73 ± 0.19	9.41 ± 0.28	14.08 ± 0.37	226.29 ± 17.07	283.74 ± 9.73	256.94 ± 7.43	298.48 ± 11.32	**2-3 ****	**2-3 ****	**2-3 *****	**2-3 *****	**2-3 ****	**2-3 ****	**2-3 ****	**2-3 ***
	3	22	1.52 ± 0.77	5.04 ± 0.22	8.30 ± 0.33	12.64 ± 0.43	175.91 ± 20.27	251.23 ± 11.55	231.68 ± 8.82	271.36 ± 13.44								

Significance of differences: *** - P≤0.001; ** - P≤0.01; * - P≤0.05.

The studies of Václavková et al. (2012) showed that pigs with a live weight at birth of less than 1000 g have a lower average daily increase over the period from birth to weaning, and those weighing more than 1500 g have the highest average daily gain. The authors found that the birth weight of pigs affected their ability to grow. Pigs with a lower live weight had lower gain during all phases of the production cycle and remain lighter until the end of the fattening period.

According to Fix et al. (2010) higher live weight and higher average daily gain in heavier pigs was a result of their greater muscle fibre count and their dominance during the suckling period.

Animals with lower live weight at birth had a reduced number of muscle fibres, an underdeveloped liver and digestive system. This was the result of slower growth during the suckling period and post-weaning period compared to pigs with higher live weight at birth (Gondret et al., 2005).

Similar to our results were the results obtained by other authors. In the study of Ambroziak et al. (2017), mean body weights at 7.21 and 56 days of age differed between group I, II, III and IV. The daily gain in group I-IV increased during the growing period (days 1-7, 8-21, 22-56). The differences between the second and third groups were small (P≤0.05), and those between the first and fourth groups were significant. The correlation coefficient for pigs in group I (lighter at birth) and group IV (heavier at birth) confirmed the relationship between birth weight and weight at 7th (P≤0.01), 21st (P≤0.01) and 56th days of age (P≤0.05), with decreasing trends in the calculations. Birth weight in group I correlated with the average daily gain from day 1 to day 7 (r = + 0.365; P≤0.01) and from day 1 to day 56 (r = + 0.291; P≤ 0.05).

Nevrkla et al. (2017) concluded in an experiment with suckling pigs, that the birth weight of the pigs influenced the growth rate before weaning, after weaning and during the fattening period. Pigs with higher live weight at birth reached slaughter weight earlier, which reduced feed consumption and the costs associated with fattening.

Rehfeldt et al. (2006) found that pigs with a lower live weight at birth grew slower and accumulated more fat until they reached pre-slaughter live weight. This was probably due to the lower myofibrillar hyperflation. The lighter pigs, according to the authors, had a lower meat quality, expressed in greater loss of water and reduced brittleness.

Ambroziak and Rekiel (2017) found a positive correlation between birth weight and weaning

weight, as well as between birth weight and slaughter weight.

According to other authors, the live weight at birth of pigs has an impact not only on their development, but also on their dropout rate and their productivity. Mortality in low-live-weight pigs was high. As the average live weight at birth increased, the percentage of surviving pigs increased. The weight at birth of piglets larger than ≥1.60 kg guaranteed a better level of gain and survival (Ambroziak et al., 2017).

According to a study by Pietruszka et al. (2017), weight at birth can influence sperm production in adult boars and can be used as selection criteria to determine replacement animals (boars). Reduced body weight in boars limits their ability to grow through puberty (up to 180 days of age), while higher live weight at birth restricts growth levels only during sperm production.

CONCLUSIONS

The addition of Neopigs Rescuemilk, Neopigs Smooth and Axclera-P in suckling pigs from birth to weaning on the 28th day had a significant positive effect on average daily gain and weaning weight. The inclusion of only two of the additives (Neopigs Rescuemilk + Neopigs Smooth) in the first group and (Neopigs Smooth + Axclera-P in the third group) had a smaller effect on the development. When including dairy supplements during the suckling period (0-28 day), birth weight should also be taken into account, which was also a significant factor in the animals' development.

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REPRODUCTION, PHYSIOLOGY, ANATOMY

Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

NATURAL AND ARTIFICIAL INSEMINATION IN SHEEP - A REVIEW

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Abstract

In the temperate zone, sheep show an annual rhythm in their reproduction with alternating estrous and anestrous periods. In both seasons, biotechnical methods for intensification of the reproductive process include synchronization of estrous and ovulation through non-hormonal and hormonal methods and insemination. In sheep breeding practice, two methods of insemination are applied - natural and artificial insemination Factors influencing the efficiency of fertilization are: breed, age, season, feeding, physical condition of sheep and rams, method of synchronization, dose of gonadotropic preparation, method of insemination, time and frequency of insemination, use of fresh, chilled or frozen semen. In both methods of insemination, it is necessary to create good organization and control. The objective of the present review is to examine and summarize the factors influencing the result of the application of natural or artificial insemination of sheep.

Key words: *sheep, natural insemination, artificial insemination, laparoscopy.*

INTRODUCTION

Most sheep breeds in the world are seasonally polycyclic. It is customary for the anestrous season in the temperate zone to begin in mid March and end in late July or early August (Hristova, 2007). Seasonal anestrous is characterized by the absence of ovulation and sexual behavior and is a major factor hindering the year-round production of sheep's milk and lamb. There are sheep breeds that do not have a pronounced seasonal anestrous, such as Dorset Horn, Romanovska, Finnish Landrace, Ile de France, Northeast-Bulgarian fine-fleece, Merino etc. Establishing the presence of normal sexual cycling activity in all seasons of the year creates a basis for intensification of the reproductive process in these breeds (Goodman, 1994). Increasing the productivity of sheep breeding is essential for the economic efficiency of herds, which is associated with improved reproduction control (Nedjraoui, 2006). Assisted reproductive technologies are used to improve the reproductive characteristics of livestock and to accelerate genetic progress. These technologies include the application of various schemes for synchronization of estrous and ovulation, programmed insemination, freezing of sperm, transfer of embryos etc. Depending on the means used to synchronize estrous and ovulation, there are hormonal and non-hormonal methods. In sheep breeding practice, two methods of insemination are applied: natural and artificial.

Natural insemination can be performed with or without estrous synchronization. The advantages of the method are: cheaper, easier to perform, minimal stress for the animals, higher fertility (lambed ewes/inseminated ewes x 100) and no special equipment and trained specialists are needed (Baruselli et al., 2018). The disadvantages are the following: no rapid progress can be expected in the flock, faster depletion of the male breeder, because his intercourses cannot be regulated, a smaller number of sheep is covered by one ram (30-40 sheep), the period of use of the brood animal is shortened, covering one sheep repeatedly and the presence of unfertilized sheep, easier spread of sexually transmitted diseases in the flock, the date of insemination and the origin of the offspring cannot be registered. In sheep breeding practice, the so-called "hand release" is applied, in which the intercourses can be controlled, the ram is protected from exhaustion, fertility is increased, the date of insemination and the origin of the offspring are registered (Tyankov et al., 2000). The rams

must be changed once every two years to avoid inbreeding.

Artificial insemination of sheep is a basic biotechnical method by which, in combination with the synchronization of estrous and ovulation, rapid genetic progress can be achieved through the use of a limited number of elite male sires (Alvares et al., 2015). In artificial insemination, fresh, chilled or frozen semen is used. According to the place of application of the sperm, are distinguished shallow cervical, deep cervical and intrauterine insemination (laparoscopic). To obtain good quality semen, it is recommended that rams perform 2-3 jumps per day for a period of 4-5 days, followed by 2 days of rest (Cueto and Gibbons, 2010). An important feature in the application of artificial insemination is the detection of ewes in estrous cycle. For this purpose, tester rams are used, which are raised separately from the sheep.

In France, more than 300,000 sheep are artificially inseminated annually, in Australia 500,000 sheep, 60,000 in Spain and 50,000 in Canada (Hernández Ballesteros et al., 2015). Artificial insemination of sheep is also well developed in Brazil, Argentina and Uruguay (Gibbons et al., 2019), while Bulgaria is one of the countries with poorly developed artificial insemination of sheep.

The advantages of the method are the following: maximum use of highly productive sires and fast transfer of their valuable hereditary qualities to a large number of offspring. 300-500 sheep can be inseminated by one ram. The date of insemination is registered, the paternity of the offspring is determined, individual selection and selection of the sheep, increase of the fecundity and twinning, protection of the animals from infectious, parasitic and venereal diseases are applied; reduction of infertility, the use of frozen semen avoids the import-export of valuable sires. Cryopreservation facilitates the long-term storage and transport of sperm. The frozen semen allows the conservation of endangered species or breeds, as well as in programs to eradicate various diseases (Tyankov et al., 2000; Amiridis and Cseh, 2012). The disadvantages of the method are: more expensive, more difficult to apply, more stressful for animals, tools, consumables and special equipment are needed, performed by qualified specialists, lower fertility, deep cervical insemination is difficult due to the anatomical complexity of cervix of sheep, reduced life expectancy of chilled sperm, the freezing procedure reduces the motility and viability of sperm, etc.

Allaouia et al. (2014) reported about fertility and fecundity (the number of born lambs (including all born lambs - live, dead and aborted)/lambed ewes x 100) in Algerian sheep breed Ouled Djellal of 86.70%, 116.54%, after natural insemination, 64% and 103% after artificial insemination.

The result of the application of natural and artificial insemination is influenced by a number of factors, such as: breed, age of sheep and rams, season, ambient temperature, farm, feeding before and after insemination, physical condition of sheep and rams, the method of synchronization, the dose of the gonadotropic preparation, the method of insemination, the period and frequency of insemination, the use of fresh, chilled or frozen semen, the place of application of semen in the female reproductive system, stress, the quality of semen, the experience of the insemination technician, etc.

RESULTS AND DISCUSSIONS

Some of the factors influencing the effectiveness of natural and artificial insemination in sheep will be discussed in more details in the present article.

The breed of sheep. The appropriate period for insemination is different for different breeds of sheep. This could be explained by the unequal period and rate of ovulation in different breeds (Fukui et al., 2010; Palacin et al., 2012), as well as differences in the physicochemical properties of cervical mucus, which can lead to difficulties in sperm movement (Kaabi et al., 2006; Richardson et al., 2011).

Karagiannidis et al. (2001) found that the optimal period for artificial insemination, after synchronization of estrous during the anestrous season in sheep of Chios, Vlachiki breeds and crossings (Chios x Vlachiki) is different. The most favourable moment for insemination of Vlachiki is the 48th and 60th hour, and for Chios and Chios x Vlachiki is the 48th and 72nd hour after removal of the vaginal sponge.

Artificial insemination administered at 60th hours after progestogen removal in Corridale sheep breed resulted in a higher fertility rate (Romano et al., 1997).

In Suffolk sheep, the optimal period for insemination was found to be 48th hours after removal of the vaginal sponge (Findlater et al., 1991). Whereas for Border Leicester × Scottish Blackface crossings, it is recommended to perform the artificial insemination at 54th -58th hours (Aitken et al., 1990). At Merino sheep it is recommended to be between 60th and 72th hours after the removal of the sponge, although the average ovulation time is the same in these two breeds (Eppleston and Roberts, 1986).

Age of animals. With age, fertility and fecundity in sheep decreases, which is associated with the presence of more reproductive disorders in older sheep, as well as the ovulation of fewer ova and lower quality than young sheep (Belkasmi et al., 2010).

In their study conducted on Churra, a Spanish milk breed, Anel et al. (2005) reported that as the age of the sheep increased, their fecundity decreased by 1.74% per year when using vaginal artificial insemination and by 2.07% when using the laparoscopic method.

Alabart et al. (2002), studied the influence of age on fertility in a total of 3819 sheep of the Spanish breed Aragonesa aged 1 to 12 years, artificially inseminated. In the present study, the authors reported a maximum fertility (56.7%) at 3 years of age, and in sheep aged 2 to 5 years the average fertility values were over 50%.

After vaginal artificial insemination with chilled semen in Chios and Lacaune sheep, Priskas et al. (2019) found that the animals of the 2nd (50.3%) and 3rd lactation (48.1%) had the highest fertility rate, which decreased with age.

According to Palacín et al. (2012) the fertility of sheep in cervical artificial insemination after the fifth birth is significantly reduced.

The age of ram affects the motility and concentration of sperm. Benia et al. (2018) reported that sperm motility was better in 3-4 year old males of Ouled Djellal breed.

A study on local rams in Bangladesh aged 1 to 4 years shows that with increasing age, sperm quality improves and stabilizes up to 3 years of age (Hassan et al., 2009). Ntemka et al. (2019) report that Chios rams maintain high sperm quality until the age of 13.

Breeding season. A characteristic feature of reproduction in sheep is seasonality. It is mainly determined by the length of daylight. Its reduction leads to the onset of the estrous period, during which the female animal is dispersed and fertilized. It is customary for the anestrous season in the temperate zone to begin in mid March and end in late July or early August. Seasonal anestrous is characterized by the absence of ovulation and sexual behavior (Hristova, 2007). Seasonal changes in temperature are of secondary importance. Fever can lead to ova death, embryonic death, and suppression of sexual reflexes (Hristova et al., 2011a).

Palacín et al. (2012) analyzed the results of 18,328 cervical artificial inseminations with chilled semen in sheep of the local Spanish breed Aragonesa. The authors reported the highest fertility in July, August, September and October (58.3%, 58.1%, 61.9% and 65.2%).

After artificial insemination in another Spanish breed (Churra), Anel et al. (2005) received lower fertility for the period July - August. The probable reason is that Churra breed, which is specialized in milk production, is more susceptible to heat stress than Aragonesa breed. The season also affects the quality of semen in male animals, which can lead to reduced fertility and fecundity (Kukovics et al., 2011).

Higher values of sperm motility were found in the breeds Mountain Corriedale, East Friesian and Copper-red Shumen during the autumn-winter period and lower during the spring-summer period (Manolov and Georgieva, 2008).

In rams of the native South African breed Zulu, Ngcobo et al. (2020) received sperm with higher volume and motility during the estrous season (0.97 ml and 92.01%) compared to the anestrous (0.72 ml and 88.69%).

During the estrous season, the inseminating ability of rams is higher (Boykovski et al., 2017). Semen obtained only during the breeding season can be successfully frozen (Manolov and Georgieva, 2008). In intrauterine insemination of sheep with frozen semen, fertility is higher during the estrous season (60%) than in the anestrous season (45%) (Dovenski et al., 2012).

The body condition of the animals. In the sheep, which at the beginning of the random campaign had an BCS (body condition score) 2.5-3.5 points, a higher number and better quality ova were reported, as well as a higher fertility and fecundity rate (Fukui et al., 2010). While in weaker animals (BCS below 2.5) the number and quality of ovulated ova decreases, embryonic growth decreases and embryonic mortality increases (Abecia et al., 2014; Ridler et al., 2017).

Sheep weight is an important factor in determining the effect of estrous synchronization. Gizaw et al. (2016) reported that sheep with BCS of 2.5-3.5 points at the onset of estrous synchronization responded best to hormonal stimulation. These animals have shown the clearest signs of estrous and have the highest fertility. Identical results were obtained by Priskas et al. (2019) in Chios and Lacaune sheep breeds, after applied artificial insemination. The authors received 48.4% and 49.4% fertility in sheep with BCS 2.5 and 3.5.

Synchronization of estrous with progestogen and/or prostaglandin. Synchronization of estrous during the estrous and anestroul season has common principles, but there are also differences arising from the unequal hormonal status. The main difference is that after discontinuation of progesterone treatment in the breeding season, a large amount of LH and FSH is spontaneously released from the pituitary gland, causing complete estrous. During the anestrous season, FSH and LH peak do not appear on their own after treatment with progesterone or its synthetic analogues, additional administration of serum gonadotropins is required. Induced estrous at rest is single (Barrett et al., 2008).

Induction of estrous by prostaglandins can only be used during the breeding season (Fierro et al., 2017). Zonturlu et al. (2011) inseminated Awassi sheep naturally after synchronization estrous with vaginal sponges impregnated with progesterone and different doses PMSG (300, 400 and 500 IU). The reported fertility is similar in all three groups and is between 80.0-82.6% and fecundity is 100%.

Metodiev and Raicheva (2011) synchronization the estrous of Ile de France sheep breed in two schemes. One with progestogen for 6 days + prostaglandin + 250 IU PMSG, when removing vaginal sponges (1 group), and the other with progesterone and PMSG (2 group), after which the animals were artificially inseminated with fresh, undiluted semen, at a dose of 0.2 ml. The authors reported fertility and fecundity of 63.64% and 142% in the first group and 43.45% and 140% in sheep in the second group. In sheep of the same breed Metodiev (2019) induces estrous through different duration of short progestogen treatments (5, 6 and 7 days) + synthetic analogue of PGF2α + gonadotropin + single natural insemination. Fertility of 54.54-63.63% and fecundity of 171.43-200% were obtained.

Almeida et al. (2018) reported that fertility and fecundity in sheep were similar after two treatments with prostaglandins at different time intervals (7, 9, and 11 days).

After treatment with prostaglandins, fertility is higher in natural insemination (Metodiev, 2017). In the case of natural coverage (by hand), Sözbilir et al. (2006) reported fertility of 60.2% in the group with an interval of 10 days and 73.3% in the group with an interval of 14 days. While in artificial insemination fertility varies from 22% to 62% (Fierro et al., 2013).

The dose of gonadotropic preparation (PMSG) used. PMSG treatment causes rapid follicle growth, increases the number of ovulated follicles and synchronization estrous and ovulation (Eekass et al., 1989), and also allows to reduce follicular atresia (Hirsfield, 1989). The dose of the gonadotropic preparation needs to be adjusted according to the breed, the physical condition of the sheep and the season (Gibbons and Cueto, 2012). During the estrous season, PMSG dose should be higher in breeds with lower fecundity and lower in breeds with higher fecundity. Breeds with "deep anestrous" require higher doses of serum gonadotropin during the anestrous season (Bonev et al., 2002).

Aköz et al. (2006) found that treatment with 700 IU PMSG in the anestrous season in Akkaraman crossings resulted in increased fertility, fecundity and twinning (100%, 86.6%, 69.2%).

Hristova (2007) reported that in Tsigai sheep breed, during the anestrous season, doses of 400-500 IU PMSG are optimal.

Following the estrous synchronization with a progestogen and injection of different doses

(300, 400, 500 and 600 IU) of PMSG at the beginning of the estrous season, in combination with vaginal artificial insemination, the highest fertility, fecundity and twinning (67.9%, 126.2% and 58.9%) was reached in Kurdi, an Iranian sheep breed, at a dose of 600 IU PMSG (Nosrati et al., 2011).

Gibbons and Cueto (2012) report that in the breeding season, the recommended dose of the gonadotropic preparation for Merinos breed is 250-300 IU and 300 IU for Corriedale and Texel breeds.

The high dose of exogenously imported serum gonadotropins (1000 IU) leads to overstimulation of the ovaries and hence the other organs of the genital system, carries risks of cystic changes, which leads to disturbances in the processes of fertilization and implantation (Bonev, 2003; Ralchev et al., 2007).

Period and frequency of insemination after estrous synchronization – The optimal time for artificial insemination after gonadotropin injection 48th hours before removal of vaginal spongesis 36th - 48th hours, according to Hristova et al. (2011) and at 48th and 60th hours when applying the same at the time of removal of vaginal sponges.

A number of authors (Menchaca and Rubianes, 2004; Jha et al., 2020) recommend that artificial insemination in sheep should be performed twice, at the 54th and 60th hour after removal of the vaginal sponges.

According to Cseh et al. (2014) the most appropriate period for single or double artificial insemination is 55th and 50th-60th hours after sponge removal, respectively.

Cueto and Gibbons (2011) found that the most favourable period for cervical artificial insemination is between 53th and 56th hours after injection of the second dose of the prostaglandin analogue. While intrauterine insemination should be performed between 58th and 66th hours after hormonal treatment.

While Metodiev (2017) administered a single natural insemination at 49th hours after the second dose of prostaglandin.

The use of fresh, chilled and frozen semen. In sheep breeding practice, fresh semen is mainly used, with fertility ranging from 70% to 82% (Donovan et al., 2001; 2004; Ehling et al., 2003). The fresh semen should be used immediately after collection, as sperm viability decreases rapidly. It is used undiluted or diluted. It can be stored at

28-30°C in a thermostatic bath during insemination for a period of 30-60 min (Gibbons et al., 2019).

In Dorper sheep breed with synchronized estrous and artificially inseminated with freshly diluted semen at a dose of 0.1 ml, Zeleke et al. (2005) obtained 75% fertility and 94.6% fecundity.

After vaginal artificial insemination of sheep in Argentina with fresh semen, with a sperm concentration between 60 and 100 million per sheep, Naim et al. (2009) reported fertility of 60%, and Prieto et al. (2011) - 70%. Cueto and Gibbons (2010) obtained identical results for the same reproductive index. The authors conducted their study with sheep in Uruguay, which were inseminated with fresh, undiluted semen, at a dose of 0.2 ml and a concentration of 100-150 million sperm.

Insemination with chilled and frozen semen in sheep breeding is more limited (Salamon and Maxwell, 2000). In chilled semen, the speed of sperm transport in the sheep's genital tract is lower than that of fresh semen (Fernández et al., 2001).

A number of authors (Naim et al., 2009; Cueto and Gibbons, 2010) reported an average fertility of 65% obtained after vaginal artificial insemination with fresh undiluted semen and 40% with chilled. While Stefanov et al. (2006) reported higher values of the indicator after insemination with cooled semen - 64.2-73.33%.

The use of artificial insemination with frozen semen is hampered by a number of factors, such as: the specific anatomy of the sheep's cervix, which is a physical barrier to sperm deposition in the uterus (Anel et al., 2005), the lower resistance of rams to semen freezing, sheep sperm are more sensitive to thermal stress than cold (Dinatolo, 2011), lack of proper cryopreservation and storage methods (D'alessandro and Martemucci, 2003; Santolaria et al., 2011; Gibbons et al., 2019).

Fertility in deep cervical insemination with frozen semen varies between 25-35% (Anel et al., 2005; Faigl et al., 2012), and in vaginal insemination it is even lower - 5-15% (Cseh et al., 2014).

Only through the laparoscopy method it is possible to deposit frozen-thawed semen in the uterine horns of sheep. Fertility in this method reaches approximately 60-75%.

When using cryopreserved semen, the ejaculate is pre-diluted in order to increase its volume and inseminate a larger number of sheep, as well as to provide a suitable nutrient medium necessary for its storage (Cueto et al., 2016).

In Australian merino sheep inseminated with fresh semen or frozen in granules or sequins, Hill et al. (1998) had fertility rates of 82.2%, 69.5% and 71.6%, respectively.

Place of sperm application in the female reproductive system - vaginal, deep cervical and laparoscopic (intrauterine).

Vaginal artificial insemination is most often used in sheep breeding because it is the easiest to apply and with the lowest equipment costs and a qualified team of specialists needed for laparoscopic insemination (Dovenski et al., 2012). In vaginal insemination, a dose of 0.1-0.2 cm^3 is injected, with 80-100 million live sperm with active translational movements.

Satisfactory results of deep cervical insemination have not yet been achieved, especially in the anestrous season and when using frozen semen. Probably the cause is the impaired transport of sperm through the cervix (Boland al., 1983). This problem can be overcome by intrauterine sperm deposition (Ishwar and Memon, 1996).

Using intrauterine insemination, the problem of the "cervical barrier" has been overcome, satisfactory fertility has been achieved by significantly reducing the number of sperm per insemination (Salamon and Maxwell, 2000). Insemination is performed based on monitoring of ovarian function, which leads to accuracy in fertilization (Yufeng, 2012). It is an alternative method of artificial insemination using frozen-thawed semen (Abdalbari et al., 2012).

Compared to vaginal at laparoscopic artificial insemination, higher fertility results were obtained (44.89% vs. 31.25%) in Churra sheep breed (Anel et al., 2005).

Taqueda et al. (2011) also reported higher fertility obtained after intrauterine, deep cervical and vaginal insemination with frozen semen - 45.8%, 25.7% and 15.4%. After deep cervical insemination with one or two inseminations, Kumar and Naqvi (2014) reported fertility of 20% and 26%, respectively. Bonev et al. (1991) achieved 75-83% fertility in laparoscopic insemination of sheep.

After estrous synchronization in Ghezel sheep, Najafi et al. (2014) applied two insemination methods - vaginal and laparoscopic. The authors received 60% fertility, 60% economic fecundity and 28.7% twinning in vaginal and 83.3%, 76.6% and 30.4% in laparoscopic insemination.

Dovenski et al. (1997) found a higher percentage of fertility and fecundity in goats inseminated intrauterine (80.95% and 182.35%) compared to vaginal inseminated (67.48% and 176.98%).

CONCLUSIONS

Establishing the links among all the mentioned factors in the different sheep breeds raised in different climatic areas is necessary for the proper regulation of the reproductive process, and hence for increasing the productivity of the flocks.

Estrous synchronization and ovulation are a key element of effective reproductive management in sheep. It allows planning and conducting the breeding and lambing campaign in a short time.

An important condition for increasing the efficiency of natural or artificial insemination is the use of rams with high sexual activity and sperm of good quality and high insemination capacity.

The highest fertility with frozen semen was achieved during intrauterine laparoscopic insemination.

In both methods of insemination, it is necessary to create good organization and control.

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THE CORELATION BETWEEN CONFORMATION TRAITS AND MILK PRODUCTION AT HOLSTEIN COWS

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Abstract

The objective of this research was to evaluate the correlation between the conformation traits and milk production at Holstein cows in Romania. It is well known the fact that milk production is influenced by many factors like: management, genetics, nutrition, season etc. But this study wants to reveal if the milk production is correlated with the conformation traits and how they influence it. In the study were taken farms from three different areas of Romania, East, South and West, also were analyzed farms of different size (small, medium and big). The animals that took part at the study were at first, second or third lactation. For all the cows were appreciated a number of 22 characters, characters that are separated in five different groups: dairy capacity, dairy character, rump, feet & legs and mammary. All the classified animals were between 20-120 days in lactation. To avoid possible inconclusive conclusion, in the study were compared cows from same area, farm dimension and number of lactation.

Key words*: conformation traits, dairy cow, milk production.*

INTRODUCTION

The milk production is an important branch of agriculture in many countries and also in Romania. Milk and dairy products have a share of approximately 30,9% of the net daily consumption of a resident of Romania, so the biggest part from animals products (INS, 2019).
In 2019, the processing units collected 1.125.300 tons of milk from national farms. That amount was not enough, in the same year being imported 128.138 tons of milk (INS, 2020).
Under this conditions, an increse of cattle effective was expected, but the opposite happened. The data from National Institute of Statistics indicate that at 1 June 2020, the number of cattle decreased with 2.4% from the total number and with 1.7% of reproductive heards. The reasons can be numerous: the lack of workforce, exploitation of unproductive animals, unsatisfactory grants, high production costs and many more. However, solutions must be found. The production efficiency of cows is closely related to milk yield in successive lactations and longevity (Sawa et al., 2013)
The factors that may influence milk production must be analyzed. In that way, the farmers can eliminate, or reduce the factors that negatively influence it and exploit the animals as profitably as possible.
In the same time, the factors that can influnce the milk production must be studied in the order to improve it. The milk production is influenced by so many factors (genetics, nutrition, management, climate, etc.). Some of them can be controlled easy, others more difficult and with great financial efforts. In addition to these factors we bring in the study another factor that can influence the milk production – the conformation traits. These traits appear earlier in life and may allow for faster selection of prolific animals (Małgorzata & Zarnecki, 2012, Madrid et al., 2014). The tool that helps us to have an overview of the herd that we are working with is the type classification action (Pașcu et al., 2018).
If at the first sight the conformation traits have nothing to do with the milk production, in reality things may be different.
Therefore, in order to put into evidence the corelation between the conformation traits and

milk production at Holstein cows, the paper present an analysis of the cows whose conformation traits were analyzed and their milk production.

MATERIALS AND METHODS

The target of the paper is to conduct a study on the correlation between the conformation traits and milk production at cows. Conformation traits have been proposed as indirect indicators for the improvement of productive and sanitarian parameters (Madrid et al., 2014).In order to elaborate the study were classified cows, by conformation traits, from nine different farms, farms located in East, South and West of the country. All the cows classified were Holstein breed.

For the cows classified were appreciated five groups of characters. The first group is *Dairy capacity*, including the traits: stature, body depth and chest witdh. The second group is *Dairy character*, where is appreciated the angularity of the animal. *Rump* is the third group, including the characters: rump angle, rump witdh and loin strength. The fourth group is *Feet & Legs* and presents the followingtraits: foot angle, rear legs - side view, rear legs - rear view, bone quality and locomotion. The last group is represented by *Mammary,* with the traits: fore udder attachement, rear udder height, rear udder width, suspensory ligament, udder depth, front teat placement, rear teatplacement, teat length, udder texture and udder tilt.

After all the traits is appreciated the animals receive a final score (Figure 1), these being ranked as follows:

- Score between 50-64 points - unsatisfactory;
- Score between 65-74 points - satisfactory;
- Score between 75-79 points - good;
- Score between 80-84 points - good plus;
- Score between 84-89 points - very good.

The final score is calculated based on the group score. Dairy capacity represents 10% from final score, dairy character 10%, rump also is 10, feet & legs 30% and mammary 40%.

In order to highlight the correlation between the conformation traits and milk production, in the study were compared the finals scores obtained by animals after classification with the standard lactation.

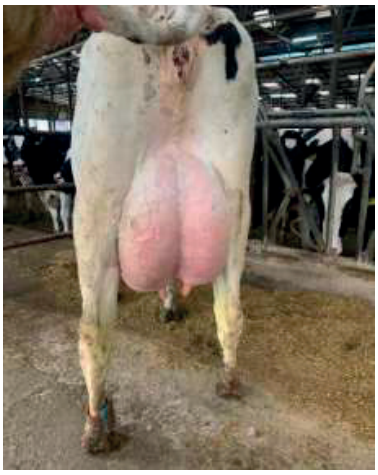

Figure 1. Cow classified as very good

The data collected from the classifier activity at HolsteinRo association have been interpreted and statistically processed building in this way the trend line.

RESULTS AND DISCUSSIONS

For the study were classified a number of 105 cows, at first, second and third lactation. All of them in the period September - November 2019. The appreciation took place when the animals were between 20-120 days in lactation. In the small farms were classified 5 cows in each farm, 10 cows in medium farms and 20 in the big farms. Next year, in 2020, the cows were weaned and standard lactation was estimate in order to make a correlation between the score obtained for conformation traits and the quantity of milk produced.

In the first phase we will present the results from the small farms.

Table 1. Small farm from South of Romania

	Conformation Score	L305 (kg)
Cow1	65	5336
Cow2	69	6044
Cow3	73	6144
Cow4	73	6152
Cow5	75	6632
X + Sx	71 ± 1.79	6061.6 ± 208.78
S	4	465.60
V%	5.63	7.68

In this farm it's easily to observe the fact that the standard lactation is bigger as the score is higher (Figure 2). For cow 1, classified as -

satisfactory the lactation was only 5336 kg. Meanwhile, cow 5 scored with 75 points, ranked - good the milk production was 6632. For cows 3 and 4, appreciated with 73 points, the lactation was almost the same 6144 respectively 6152. The average for the conformation score is 71 points, with an average error of 1.79 points. The average for the milk production it's only 6.061 kg.

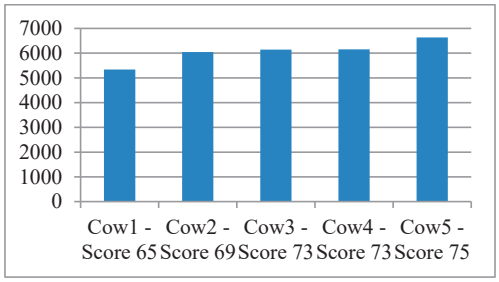

Figure 2. The dynamics of milk production in relation with the conformation score (small farm - South area)

Table 2. Small farm from East of Romania

	Conformation Score	L305 (kg)
Cow1	72	7092
Cow2	74	7235
Cow3	74	7572
Cow4	78	8116
Cow5	79	8571
X + Sx	75.4 ± 1.33	7717.2 ± 277.56
S	2.97	618.97
V%	3.93	8.02

In Table 2, we can see that the average for conformation score was higher than in Table 1, being 75.4 points. Corelated with that we have the average for milk production equal with 7717 kg.

The farm located in the East area (Figure 3) presents better conformation scores that the one from the South and also bigger milk production. Cow number 5, scored 79 points, ranked - good, produced 8571 kg of milk on standard lactation. The difference between the cow1, with 7092 kg and cow 5 with 8571 kg is approximate 1.500 kg and 7 points at conformation score, for the cow 5. Cow 4, classified also as - good, but with 78 points produced 8116 kg, not significantly differences from cow 5 (Figure 4).

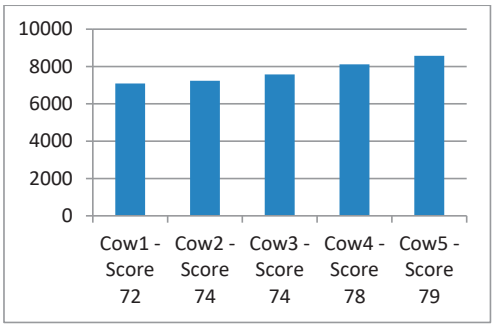

Figure 3. The dynamics of milk production in relation with the conformation score (small farm - East area)

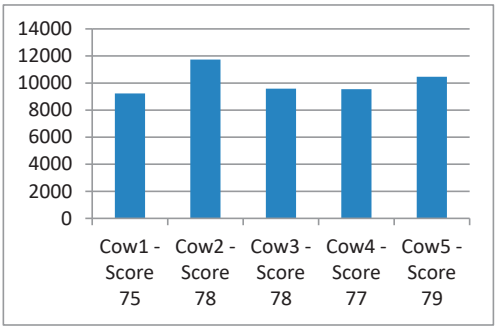

Figure 4. The dynamics of milk production in relation with the conformation score (small farm - East area)

In the third small farm are not significant differences in the conformation scores obtained, all cows belonging to the class - good. The lowest score was obtained by the cow 1-75 points and the biggest by the cow 5-79 points. However, at milk production the biggest differrence was registered between cow 1 and cow 2, with 9228 kg, respectively 11737 kg. Cow 3, appreciated also with 78 points produced only 9584 kg of milk in standard lactation. But of course, how we mentioned before, all the cows from this farm are in the same class of conformation traits. Therefore, it's difficult to elaborate a conclusion extrapolating from this farm.

The farm from the West of Romania has the biggest average for conformation score by all small farms, 77.4 points (Table 3).

All the animals that took part to the study from the small farms were at their first lactation.

Further, we will present the results from the farms with medium dimension. We make the mention that in this type of farms the animals studied were at second lactation. The average for the milk production it's also the highest, 10111 kg (Figure 5).

Table 3. Small farm from West of Romania

	Conformation Score	L305 (kg)
Cow1	75	9228
Cow2	78	11737
Cow3	78	9584
Cow4	77	9544
Cow5	79	10463
X + Sx	77.4 ± 0.68	10111.2 ± 456.58
S	1.52	1018.17
V%	1.96	10.07

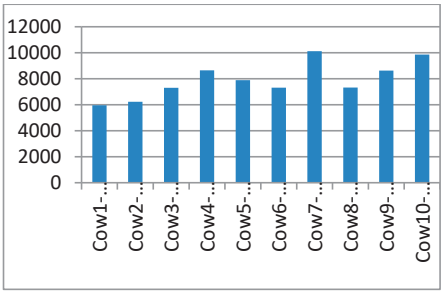

Figure 5. The dynamics of milk production in relation with the conformation score (medium farm - South area)

The lowest conformation score in this farm it's obtained by cow 1, 60 points, ranked – unsatisfactory. The milk production obtained by this cow it's only 5962 kg. At the other pole, we find cow 7, ranked as - good plus, with 80 points. The cow 7 recorded 10116 kg milk production per lactation. The animals 5 and 9, scored the same, with 69 points registered 7891 kg milk, respectively 8630, a difference of 739 kg. All the results can be seen in the Table 4.

Table 4. Medium farm from South of Romania

	Conformation Score	L305 (kg)
Cow1	60	5962
Cow2	64	6227
Cow3	65	7308
Cow4	75	8644
Cow5	69	7891
Cow6	68	7310
Cow7	80	10116
Cow8	67	7324
Cow9	69	8630
Cow10	75	9861
X+Sx	69.2 ± 1,88	7927.3 ±440.27
S	5.96	1391.26
V%	8.61	17.55

In the first medium farm, we have an average by only 69.2 points for conformation score. Corelated with an average by 7927 kg for milk production, but with a standard deviation pretty big. Also, we have a coefficient of variation big, 8.61%.

Two other cows are classified the same, cow 4 and 10, the first one produced 8644 kg of milk, the second one 9861.

As a summary, in this farm the situation is: two cows ranked unsatisfactory, with an average of 6094 kg milk production, five cows ranked as satisfactory, with an average of 7692 kg of milk, two cows ranked as good with an average of 9252 kg of milk and one cow ranked as good plus with a production of 10116 kg of milk.

It's obviously the fact that the biggest production was obtained by cow 3, scored 81 points, on the second place, after lactation it's cow 4, scored 79 and after that cow 8 also with 81 points (Figure 6).

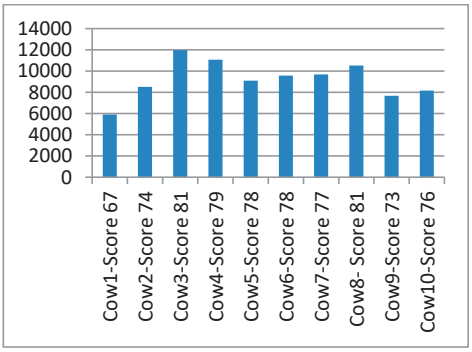

Figure 6. The dynamics of milk production in relation with the conformation score (medium farm - East area)

In the medium farm, from the East of Romania, we have three cows ranked as satisfactory, with an average of 7362 kg of milk, five cows with an average of 9519 kg of milk and two cows with an average of 11245 kg of milk (Table 5).

The average for the conformation score is 76.4, a pretty good one. The milk production presents an average by 9217 kg, also a good one.

The last medium sized farm is from the West of the country. In this farm we identified a cow ranked as very good, cow 4. This cow produced on the standard lactation a quantity of 9066 kg of milk. The animals 1 and 9, scored with 70 points have the smallest production 5519, respectively 6268 kg of milk. At cow5 and cow10, ranked as satisfactory, with 73 points we

recorded a similar lactation 7256 kg and 7795 kg of milk. All the results are available in the Figure 7.

Table 5. Medium farm from East of Romania

	Conformation Score	L305 (kg)
Cow1	67	5902
Cow2	74	8508
Cow3	81	11973
Cow4	79	11072
Cow5	78	9102
Cow6	78	9578
Cow7	77	9687
Cow8	81	10517
Cow9	73	7677
Cow10	76	8156
X+Sx	76.4 ± 1.33	9217.2 ± 558.97
S	4.22	1766.36
V%	5.53	19.16

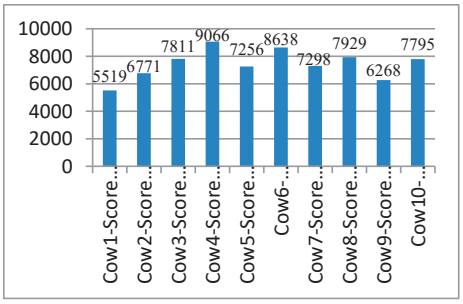

Figure 7. The dynamics of milk production in relation with the conformation score (medium farm - West area)

The last type of farms studied are the big farms, at their level we appreciated cows at third lactation. In the Figure 8 can be observed the data obtained from the farm located in the South area.

If so far the biggest milk production was obtained by the cow with the higher score, in this case things are different. Cow 9, scored 78 points has a production of 14043 kg. Cow 19, ranked as - good plus, with 81 points has a production of 13150, the second from the group. According to the distributions on conformation classes we have the next situation:

- 6 cows ranked - satisfactory, with an average of 8948 kg;
- 13 cows ranked - good, with an average of 11088 kg;
- 1 cow ranked - good plus with a production of 13150 kg.

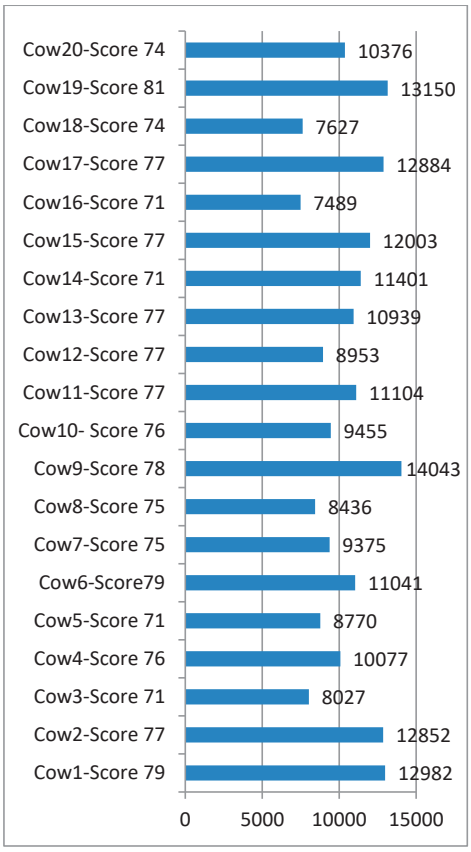

Figure 8. The dynamics of milk production in relation with the conformation score (big farm - West area)

Figure 9 presents the results from the big farm from the East of the country.

The lowest production was obtained by cow 12, only 7180 kg, cow 8 scored also with 72 points produced 10404 kg of milk.

Cow 10, ranked - very good, with 88 points produced 16123 kg, in time that cow 4 also with 88 points produced 14890 kg of milk.

The last farm presents a situation more balanced, there are no big differences between the scores obtained and the milk produced. The cows with the biggest score are 12 and 17, with productions of 8364 and 9656 (Figure 10).

Grouped by class we have 15 cows ranked - good, with an average of 8366 kg and 5 cows ranked as good plus with an average of 9372 kg of milk.

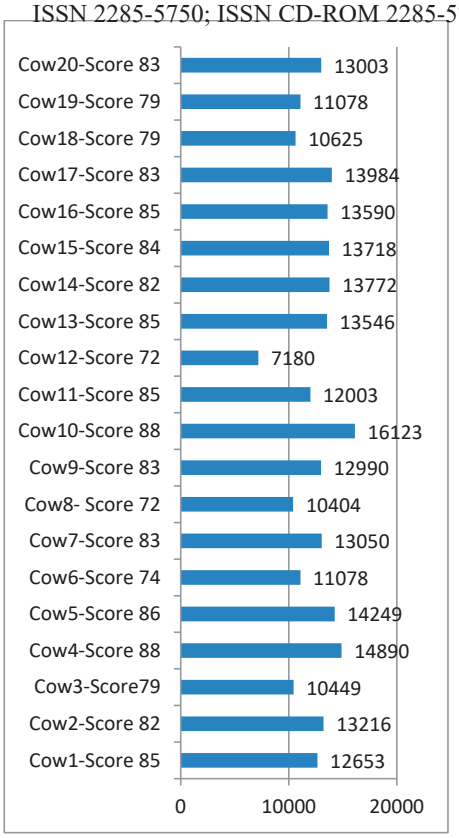

Figure 9. The dynamics of milk production in relation with the conformation score (big farm - East area)

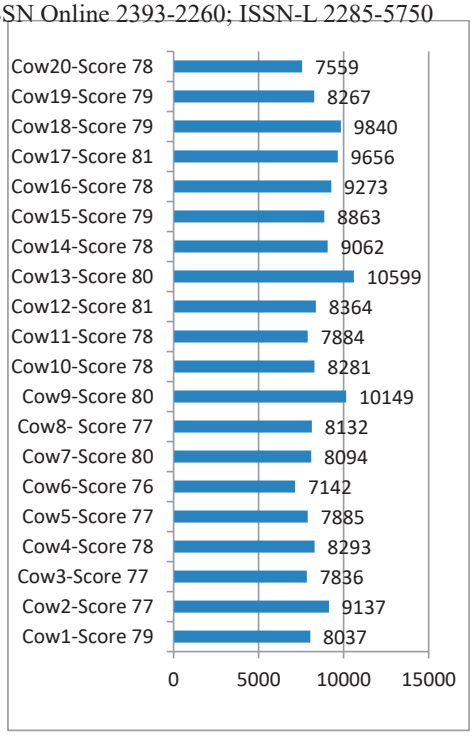

Figure 10. The dynamics of milk production in relation with the conformation score (big farm - West area)

CONCLUSIONS

The lowest milk production was always obtained by the cows ranked unsatisfactory or satisfactory.

In small and medium - sized farms (where were classified cows at first and second lactation) the biggest production was obtained by the cows with the highest score.

In the big farm (where were classified cows at third lactation) the biggest production was not always obtained by the animal with the highest score.

If we analize the data in relation conformation class - milk production we observe that in all situation as higher is the class as higher is the production.

Therefore a cow ranked as very good/good plus after the conformation traits will produce more milk that a cow ranked unsatisfactory. We can use conformation traits appreciation when we want to make early reforms.

ACKNOWLEDGEMENTS

This research work was carried out with the support of Holstein Ro association and member farms.

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INFLUENCE OF SOME EXTERNAL FACTORS ON SPECIFIC CHARACTERISTICS OF SEMINAL MATERIAL FOR KARAKUL DE BOTOȘANI BREED RAMS

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Abstract

In order to objectively assess the influence of external factors, the observations were made both in normal breeding seasons and in the off-season, the aim being to obtain conclusive data necessary for a real understanding of specific aspects of reproductive physiology in adult rams. The biological material was represented by breeding rams belonging to the Karakul de Botoşani breed located at the Research and Development Unit for Breeding Sheep and Goats Popăuți - Botoșani. In order to evaluate the mode of influence and the differences due to the experimental factor, the applied protocol aimed to expose the rams to the same effect of photoperiodicity for two consecutive seasons placed in atypical periods. The volume of ejaculate was a basic objective in the applied research, the final results indicating that from rams exposed to a photoperiod placed in the off-season has a lower average value by only 5.04% compared to the volume harvested from rams active in normal season and with over 75% more than the level harvested from those at sexual rest. Differences were also reported in the case of the analysis performed for the determination of the quality indicators of the harvested semen, respectively acidity, density and mobility. The obtained results highlight the existence of multiple factors which, due to the intensity of their influence, can induce changes in the basic characteristics of the semen.

Key words*: external factors, photoperiod, ram, reproduction function.*

INTRODUCTION

The role and influence of different natural factors on the way heat is manifested in females was a more frequently approached topic, while the effect of the same factors on the reproductive function in rams is a subject with a lesser approach.

For a more complex approach to the research objectives, the observations were made in normal breeding seasons but also in the off-season, the aim being to obtain conclusive data necessary for a real understanding of the physiology of reproduction in adult rams.

The obtained results highlight the existence of multiple factors which, due to the intensity of their influence, can induce changes in the basic characteristics of the semen. Of all these, the most important are: nutrition, maintenance conditions, presence of females, geographical location, age, testicular characteristics, libido and management system, an aspect highlighted in some scientific articles in the literature (Nowakowski et al., 1994; Madani et al., 2009; Kridli et al., 2004; Zamiri et al., 2005), but photoperiodicity and race seem to be the main factors influencing the activity of seasonal reproduction.

In this context, conducting research on the influence of natural factors on the main characteristics of semen aims to highlight the ways that can be used to optimize them and other aspects that could be generated by climate change.

MATERIALS AND METHODS

The biological material was represented by breeding rams belonging to the Karakul de Botoșani breed located at the Research and Development Unit for Breeding Sheep and Goats Popăuți - Botoșani.

In order to meet the research objectives, the experimental protocol applied a program to

induce the manifestation of sexual activity in rams in the off-season, based on the following steps:

a. long days simulation, in this case the long day was considered the one with more than 12 hours of light, corresponding to the months of late spring and the beginning of summer;

b. short days simulation, in which case a short day was considered the period in which the rams were kept in the light for less than 12 hours of a day, corresponding to the autumn-winter months.

In order to be able to establish the differences due to the experimental factor, the applied protocol aimed to expose the rams to the same effect of photoperiodicity for two consecutive seasons placed in atypical periods in an area located at northern latitude of 47.76 and longitude of 26.69, respectively in the interval March 10 - May 20, when the ratio between light and darkness is close to or greater than 1/1, and the possibility of late, semi-late and semi-early breeds to manifest sexual cycles is reduced.

At the beginning of each experimental period in the two consecutive years, for 15 days the rams were subjected to a special light program, because it is known that during the 24 hours of a day there is a period in which the animals are more sensitive to light. This period is called the photosensitive phase and is naturally placed 16-17 hours after sunrise, which is considered a landmark of circadian rhythm. Through this mode of action, a progressive exposure of the rams to a total light duration of about 16 hours was achieved, corresponding to 22nd of June.

From that moment on, in order to have conclusive results that could argue the practical possibility of intensifying the reproductive activity in rams, they were subjected to a program of gradual control of both the temperature and the duration of daylight hours of a day.

In the first 17 days there was a gradual increase in the duration of temperature and light until reaching daily values between 26 and 28°C and 15 hours of light, representing the average multiannual values corresponding to 22nd of June, after which we gradually reduced the two parameters, so that after another 18 days to ensure the exposure of batches of rams at an average temperature of 18°C and a duration of 10.45 hours of light, representing the multiannual average values for 15th of October.

From the moment that both parameters were reached, the second stage of the research was passed, namely the collection of semen, and the performance of qualitative and quantitative analyzes of semen.

Parameters that characterize the quality and quantity of semen were evaluated by specific laboratory methods and data processing was performed by methods accepted by the experimental technique.

The non-parametric Mann-Whitney U Test was used to highlight the significance of the differences between the characters analyzed at different times of the year in rams in different conditions in terms of sexual activity.

RESULTS AND DISCUSSIONS

In this species, as in goats, reproduction is determined by the genotype/environment interaction, being largely influenced by the photoperiod, meaning the duration of daylight, to which is added the influence of other factors such as: diet, temperature, humidity, social factors, etc. (Pascal et al., 2008).

Regarding the ratio between light and dark, the effect is received by sheep in the eyes through the retina and is transmitted by nervous system to the pineal gland (epiphysis) which secretes the hormone melatonin (Pascal et al., 2009).

The activity of the hypothalamus and pituitary gland, but also the duration and amount of secreted melatonin, corroborated with the actual dark period, exerts a major influence in the development of sex hormones, meaning follicular stimulation (FSH) and lieutenant hormone (LH) (Andersson et al., 2005; Barid et al., 1981; Cahil et al., 1980; Pascal et al., 2009; Thwaites, 1982).

In sheep and goats, the influence of this phenomenon is major in both sexes from herds in areas located in the northern hemisphere. In this area, reproduction takes place in autumn, and the intensity of the onset sexual cycles is mainly due to the decrease in the intensity of daily light, thus being considered animals that have a negative photoperiod (Delgadillo et al., 2014). The explanation for this behavior would be that shortening the duration of daily light causes the pineal gland to synthesize and

secrete melatonin, which stimulates the hypothalamus, pituitary gland, ovaries and testicles, with positive effects on reproductive activity.

The assessment of the main physical and biological properties of the semen harvested from rams subjected to photoperiodicity, sexually active during the normal breeding season and respectively at sexual rest, was performed for each period considered, the average values being presented in Table 1 and Figure 1.

Table 1. The main biophysical properties of ram semen according to the natural breeding season

Season/ Condition of rams	n	Biophysical behaviors of seminal liquid			
		Volume (ml)	Sperm reaction pH	Density	Mobility (%)
Exposed to photoperiod	8	1.93±0.02	6.71±0.11	1.09±0.01	83.47±0.05
Sexual rest	8	1.08±0.01	5.82±0.09	1.03±0.10	78.99±0.09
Normal season	8	2.04±0.05	6.95±0.20	1.11±0.07	82.48±0.07

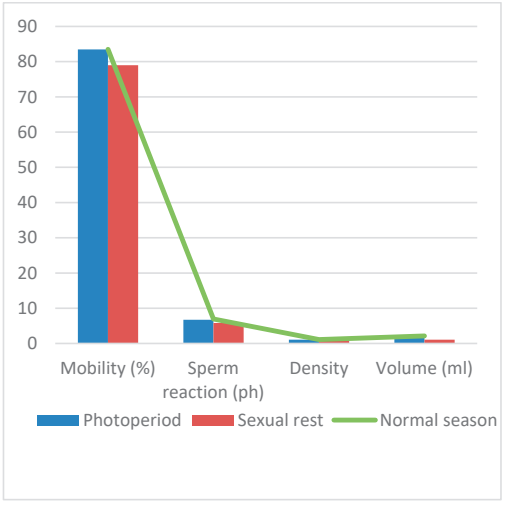

Figure 1. The dynamics of ram's semen main biophysical properties in relation to the natural breeding season

The ejaculate volume was an important objective through which we wanted to verify whether from a quantitative point of view there are differences in the volume of the ejaculate under the influence due to the conditions of the two calendar periods.

In the literature it is specified that in breeding males in the normal season of activity the volume of ejaculate had average values between 1 and 1.5 ml (Luca, 1972) and in males in sexual rest the volume of sperm is lower. Regarding the volume of ejaculate, Kuznetsov quoted by Luca (1972) shows that in rams there were situations in which the level of ejaculate exceeded 6 ml.

Research has shown that there are differences in the volume of semen harvested from rams depending on certain times of the year. Based on the determinations performed, it was observed that there are differences in the volume of ejaculate. The level of ejaculation was higher in the case of harvests performed from rams during the breeding period (2.04 ± 0.02 ml) and who had sexual activity during the autumn season and only 1.08 ± 0.01 ml in those during the sexual rest corresponding to January and February.

In the case of males exposed to the influence of light, the level of ejaculated collected had an intermediate volume of the first two situations, the average level being 1.93 ± 0.02 ml. In the case of harvests from breeding rams during the breeding season, but carried out in the normal season, meaning September-October, the volume of ejaculate was 2.04 ± 0.051 ml, and it was 47.05% higher compared to the volumetric level recorded in the case of harvests performed from rams at sexual rest and by only 5.39% compared to the volume of semen harvested from rams that were exposed to the influence of temperature and duration of light during the off-season (May-June). This difference was statistically significant for $P<0.01$.

However, other studies carried out in areas with a different climate than in the North-eastern part of Romania show that the ejaculation volume was 1.23 ± 0.31 ml being more influenced ($P<0.0001$) of the season and in very small measure of age ($P<0.84$) of rams (Barid et al., 1981).

All these values confirm that in rams subjected to the influence of photoperiodicity, the testicular secretory activity takes place in almost normal conditions and in the situation when the reproductive activity is placed in the months considered to be in the off-season.

This aspect is very important when in some farms is applied an advanced management of sheep breeding. In this case, the application of a controlled program of exposure of breeders to

the influence of light and temperature can change the general condition of rams because through photoperiodicity can stimulate the intensification of sexual activity and their use in off-season breeding.

The economic importance of the data obtained for this indicator is also ensured by the fact that the volume of ejaculate obtained from rams stimulated by external factors reaches the level obtained during normal activity. At the same time, the practical importance is obvious because when a reproductive technology based on artificial insemination is applied, the number of sown females increases with the resulting doses per ejaculate, even when the rams exposed to the influence of temperature and light have a lower ejaculate volume. This aspect does not affect the fertilizing capacity of the semen even if by reducing the volume of ejaculate there is a reduction in the total number of sperm, and the final results of insemination do not decrease significantly, an aspect reported in other specialized bibliographic sources (Nacu et al., 2011; Davis et al., 2001; David et al. 2007).

In a similar study performed on the breeders of the Merino de Palas undergoing an experimental treatment based on the influence due to photoperiodism on the reproductive characters, an average ejaculate level of 1.91 ± 0.021 ml was obtained, and from those at rest and in natural season the volume was 0.98 ± 0.06 ml and 1.94 ± 0.03 ml, respectively (Moise et al., 2015).

Regarding the volume of ejaculate from rams in the natural breeding season Kuznetov quoted by Pascal (2015) states that there are situations in which the ejaculate level can exceed 2 ml, sometimes even higher than 6 ml.

Sperm acidity or reaction is assessed by determining the pH value and may be influenced by the concentration of semen fluid in the sperm. The higher the sperm density, the more the pH tends towards acidity, an aspect determined by more intense metabolism but also by the greater accumulation of lactic acid in the seminal fluid.

Regarding the values of this character but specific to ram sperm, in the literature it is specified that in males in the normal breeding season the pH has average values of 6.8 (Luca, 1972; Nadolu et al., 2007; Pascal et al., 2008; Thwaites, 1982; Zarazaga et al., 1997).

The evaluation of this indicator is important because the duration of sperm motility proved to be pH dependent. Relatively longer periods of motility occur mainly at a range of pH levels between 5.8 and 6.4 with a maximum placed at an average value of 6.26.

In the determinations made, differences were found regarding the reaction of the semen harvested from the rams at different times of the year and under different conditions in terms of sexual activity.

It should be noted that the determined average values are close only to the groups of ram's subject to the influence of the photoperiod and those in the normal season of reproductive activity. Thus, it was found that in the case of semen harvested from rams subjected to a photoperiod, the acidity records an average value of 6.71 ± 0.11. Samples collected at the time when the breeding activity took place in the normal season from the same ram, the average acidity was 6.95 ± 0.20. This aspect allows us to conclude that there is an almost constant relationship between the epidermal contents and the secretions of the secondary glands, a sign that the spermatogenesis process evolves normally in rams under the influence of natural factors and their optimization at other times of the year.

The statistical processing of the data does not show that the difference between the average values obtained in the two periods is not significant for the statistical thresholds taken into account.

In contrast, when determining the acidity of semen harvested from rams during sexual rest, the average pH was 5.82 ± 0.09. Placing average values well below these limits is undesirable because at low pH sperm motility is reduced and has a negative effect on their viability.

Density and mobility are important indicators of assessing the quality of semen because the act of fertilization depends on mobility and the number of viable sperm.

Analyzing the specific weight of sperm in relation to the interrelationships between the physical properties and the biological value of semen, it is found that a specific weight of

semen corresponds to a particular density and mobility of sperm (Andersen et al., 2005).
After Lindhal and Kihlstrom, quote Pascal et al. (2008) the specific weight of sperm also depends on the ratio between mature (heavier) and immature (lighter) sperm in the sperm fluid. Other studies state that sperm-specific weight is directly influenced by osmotic pressure and is directly related to the cryoscopy point of sperm (Mann, 1960).
The sperm density assessed under a microscope is quite subjective and greatly influenced by the experience of the examiner. Due to this fact, in laboratory practice determinations are made by counting the spermatozoa in the ejaculate to be analyzed or other indirect procedures by which the number of sperm in a sperm volume can be established as correctly as possible. The number of sperm can be reported in millions per microliter, or in billions per ml of sperm. At the same time, it is mandatory to establish the correct degree of semen dilution (Pascal et al., 2008).
When assessing dignity, the results obtained are placed in a narrower range in rams during periods of ongoing sexual activity, the differences not being statistically significant. Based on the average values determined from semen samples collected from rams under the direct influence of temperature and light exposure time, the main density value was 1.09 ± 0.01 and for those with sexual activity during the normal season the density increases to 1.11 ± 0.07.
The research carried out wanted a comparison of the average values specific to this character in rams in conditions of intense sexual activity carried out at different times of the year. In the case of analysing this character on the semen harvested from rams during sexual rest, the average value was 1.03 ± 0.10.
Mobility is one of the quality indicators that are used to analyze the basic characteristics of semen specific to any animal species. Normal sperm are able to move quickly, in a straight line, by undulating movements of the tail. Only sperm with active linear motion participate in fertilization.
Simultaneously with the assessment of mobility and concentration, observations are made regarding the energy of movement of sperm. On the samples analyzed from the three groups of rams that were in sexual periods placed at certain intervals of the year or in those at sexual rest, the sperm mobility registered some differences of the average values. In the group of rams during sexual activity, the average value was 83.47 ± 0.05 in those who went through an off-season stimulation program and 82.48 ± 0.07 in rams who had sexual activity during the normal breeding season. In contrast, in the rams that were in the period of sexual rest, the mobility had average values of 78.99 ± 0.09. Between this average value and those determined for the two groups of rams that had sexual activity in the normal season and in the off-season, it is found after applying the non-parametric Mann-Whitney U Test that the differences are significant for $P<0.05$.
The existence of differences between the average values of rams in different conditions of use, but also the approximation of data for density and mobility to samples collected from rams in the natural breeding season and those subjected to photoperiodicity allows us to say that by optimizing light and temperature in atypical seasons the quality of semen has characteristics close to the values determined in those in full season of reproductive activity.
Based on the data obtained from the evaluation of the semen quality from breeding rams, it can be stated that the exposure of breeders to a program to simulate temperature and light duration during off-season has a positive influence on the main characteristics of sperm fluid.

CONCLUSIONS

In case of harvests from breeding rams during the normal breeding season, i.e. September-October, the ejaculate volume was 2.04 ± 0.051 ml, being 47.05% higher than the volumetric level recorded in the case of harvests from rams at sexual rest and only 5.39% compared to the volume of semen collected from rams that were exposed to the influence of temperature and light duration during off-season periods (May-June).
When analyzing the acidity of the semen, differences were found regarding the reaction of the semen harvested from the rams at different times of the year and activity.

The determined average values are close only to the groups of rams subjected to the influence of the photoperiod (pH = 6.71) and those in the normal season of reproductive activity (pH = 9.65)

Regarding the differences between the sperm density of semen, it is found that in the samples collected from rams after photoperiod treatment and from those in the normal breeding season the average values of specific gravity are very close, meaning 1.039 in those subjected to photo-periodism and 1.038 for those who work in the natural season.

Based on the data obtained from the evaluation of the quality of semen from breeding rams, it can be stated that the exposure of breeders to a program to simulate temperature and light duration during off-season has a positive influence on the main characteristics specific to semen quality.

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MONITORING OF REPRODUCTION INDICES AND THEIR INTERRELATIONSHIPS WITH MILK PRODUCTIVITY AT HOLSTEIN COWS OF DIFFERENT ORIGIN

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Abstract

Breeding indices and their interrelations with milk productivity were studied with the Holstein cow populations, of different origins (Dutch, German and French). The value of the main reproduction indices in the investigated populations significantly exceeds the maximum allowable limits. The shortest SP is at Dutch Holstein cows, the German exceeds the first by 7.1 days, the Holstein-Prim, French cows - by 32.9 days those of Dutch and by 5.8 days the cows of German origin. The longest DL has Holstein-Prim, French cows, exceeding by 66.4 days the Dutch, by 35 days the German, which in their turn exceeds, Holstein, Dutch by 31.4 days. The CI exceeds the maximum allowable value (365 days) forming the following string: Dutch Holstein (+75.7 days) < German Holstein (+99.9 days) < French Holstein-Prim (+136.4 days) and the RCC of cows - Dutch Holstein (0.87) > German Holstein (0.82) > French Holstein-Prim (0.81). Between the RCC and the indices of milk productivity on total lactation were established strong and very strong negative correlations and mostly negative from medium to very weak with the same indices on normal lactation.

Key words*: calving interval, coefficient of reproduction capacity, correlation, dry period, service-period*.

INTRODUCTION

Cattle take a primordial place in providing the products needed for the consumption of the population and in the agricultural economy, that is why the species deserves concern and support for size, performance and competition both now and in the future.

With the radical reshuffles in the ownership structure of livestock in favour of the private sector, there have been a number of shortcomings and differences, such as the practical decrease in livestock, the change of technical actions in the genetic improvement of livestock populations, official control of productions, testing of breeders by the quality of their lineage, etc. that have also been deeply felt in the cattle branch. Research carried out in recent years, on this sector in the Republic of Moldova, shows a slow decline in the herd. At the same time, using advanced growth and exploitation technologies, the productivity of specialized dairy cattle production, increase. The breeding parameters (fecundity, birth rate, calving interval, etc.) significantly influence the rate of breeding of the animals and the rate of genetic progress, the level of production, the genetic structure of the populations, the state of animal health and the economic efficiency of this branch. For this reason, the maximum use of cow's reproduction capacity is among the most important conditions that determine the high efficiency of specialised cattle for milk production. The genetic potential of productivity can only be achieved under a high level of reproductive function (Hansen, 2002; Baimishev et al., 2007; Azarova et al., 2009; Lobodin, 2010; Gritsenko, 2016).

Optimization of reproductive function is one of the key factors influencing the realization of the genetic potential of high cow's milk productivity. According to data from the literature milk production, but also its quality is influenced by various factors (Laben, 2000; Matsoukas and Fairchild, 2005; Frolova et al., 2014), but not least the value of the breeding indices (Kononov, 2013).

Although the literature has a rich arsenal of data concerning the interrelationships between milk productivity at cows and breeding indices (Mayer, 2006; Maslov, 2007; Firsova et al., 2012), however, so far, there is no single opinion on this problem, remaining in the sights of researchers and practitioners. Since

the genetic material of the Holstein breed introduced into the republic is of different origin (German, Dutch, French), and the capacities to adapt to the pedoclimatic conditions of the Republic of Moldova are different, we present the results of monitoring of breeding indices and the interrelationships between these and the performance of milk production, at the populations of cows of the Holstein breed of different origin.

MATERIALS AND METHODS

It was experimenting with cow populations of Dutch Holstein exploited in the production activity course of Limited Liability Company (LLC) "DokSanCom", district Ceadir-Lunga, two populations of Dutch and German Holstein cow exploited on the course production activity of Joint-Stock Company (JSC) "Aydyn", Comrat and with the French Holstein-Prim population exploited in the production activity course of Limited Liability Company (LLC) “Total Gnatiuc”, district Glodeni, during the period 2017-2020 years.

The study was targeted the assessment of the duration of breast restenosis (BR), service-period (SP), calving interval (CI), duration of lactation (DL), reproductive capacity coefficient (RCC) and interrelationships of reproduction indices depending correlation coefficient between the coefficient of use of reproductive capacity at cows and milk (M) productivity, duration of lactation (DL), milk fat (F) and overall fat (OF) on total lactation (TL) and normal lactation (NL) in the respective animal populations.

Statistical processing of the experimental results was carried out computerized, by mathematical analysis of biological phenomena, according to the program "Microsoft Excel 210".

Interpretation of the value of the correlation coefficient:

- r = 0 → there is no correlation;
- r = +/- 1 → the correlation is perfect;
- r > 0.4 → good correlation;
- r [0 – 0.2] → very weak correlation;
- r [0.2 – 0.4] → weak correlation;
- r [0.4 - 0.6] → reasonable correlation;
- r [0.6 - 0.8] → high correlation;
- r [0.8 - 1] → very high correlation.

RESULTS AND DISCUSSIONS

The capacity of females for milk secretion is linked with the physiological dominance of the perpetuation of the species and the feeding of progeny. With regard to milk cattle, if the female for some reason is devoid of the ability to procreate, the ability to secretion lactate is also inhibitory.

Therefore, the more offspring a cow produces, the more milk secretion will be stimulated and the more milk it will produce throughout the cow's life. So, the primary task that stands before the breeders is to obtain from each cow during the calendar year a calf.

The breast restenosis is one of the main moments in milk cattle mining technology. Namely, during this period takes place the foundation of well-being health of the mother and the product of conception, the favorable development of parturition and the triggering of successive lactation.

The duration of breast restenosis depends of the duration of lactation and the amount of milk on the previous lactation, age, expected milk productivity, level and quality of nutrition. Theory and practice reveal that the breast restenosis of less than 30 days brings losses of about 20% of milk per lactation.

When its duration is 30 to 40 days the losses will be limited to 10% in the following lactation, compared to its duration of 45 to 60 days (Boriskin et al., 2005; Lavelin, 2009).

In Figure 1 we present the results of the study of the breast restenosis duration at cows of the Holstein breed, different origin.

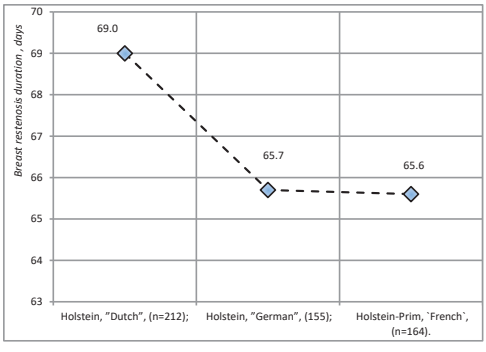

Figure 1. Characteristic of breast restenosis duration at Holstein cow populations, different origin, exploited under the conditions of the Republic of Moldova

The optimal duration of breast restenosis is 60 days, and the deviation in one or another duration has negative consequences, resulting in a decrease in milk productivity.

Analysis of the obtained data attests to the increase in breast restenosis at Holstein cows compared to their optimal value, regardless of origin. Thus, at population of Holstein cows of French and German origin the value of the examined index being similar, exceeds the allowable duration by 5.6 days (+9.2%).

The biggest gap was established in the Holstein population of cows originating in the Netherlands, taking over the optimal value by 9 days (+15.0%). Compared to the populations of french and german origin, the difference is 3.4 and 3.3 days (+5.0 – 5.2%), being non-authentic.

The reproduction of the calf includes two periods: gestation and the period from calving to fecund incemination - defined service-period. Since the duration of gestation at cows is a relatively constant value, equal on average with 285 days, the number of calves obtained from cows, per year and/or the entire period of exploitation, depends on the duration of the service-period. The duration of the service-period influences the milk production of cows, primarily as a factor determining the duration of lactation and the period of instalation of gestation. Once the increase in its duration, correspondingly decreases the birth rate to 100 cows per year, reason why it is recommended that the cows be fertilized in the first 2 to 3 months after calving, this being the main condition of increasing the rate of breeding of the herd in the household and increasing the economic efficiency of the branch (Artyukh et al., 2004; Perfilov, 2009).

Below (Figure 2) we present the results of the monitoring of service-period duration.

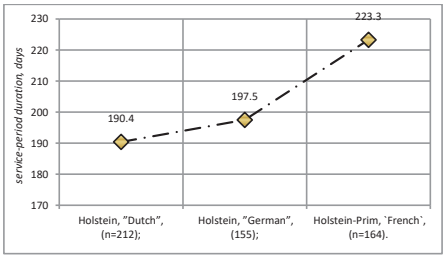

Figure 2. Service-period feature at Holstein cow populations, different origin exploited in RM

The presented results show, that compared both with the maximum allowable duration and between the populations of cows of different origin are attested to higher values of the service-period.

The best results were established in the Dutch Holstein cows. At German Holstein cows population the service-period duration is increasing vis-à-vis premiums with an inauthentic difference of 7.1 days (+3.8%). The results obtained at Holstein-Prim, French origin are the highest, exceeding with an inauthentic difference of 32.9 days (+17.4%) Dutch and 5.8 days (+13.1%) Holstein cows, German origin.

Starting from the known fact that the permissible limits for the duration of the sevice-period, it is recommended to be according to the level of milk production at cows and is considered to be:

- 45 days, for those with low milk production;
- 60 days, for those with average milk production;
- 80 days for those with high yields and
- up to 120 days for cows with record milk production.

Even so, in our case the service-period duration prevails 1.9 times the maximum allowable value for cows with very high milk production. Thus, it is obvious that cows are sown very late after calving.

Investigations with dairy cows confirm that the best results of their use are obtained when the duration of lactation is within 270 to 305 days. In the following figure we present the dynamics of lactation duration in Holstein populations of different origin (Figure 3).

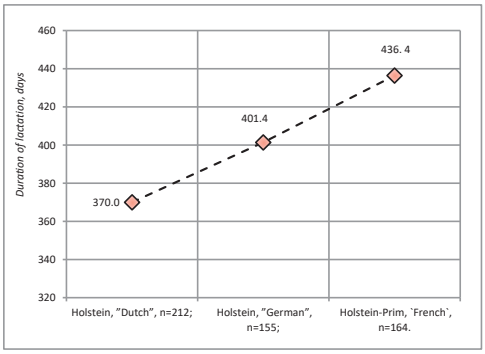

Figure 3. Duration of lactation at Holstein cow populations, different origin, exploited in RM

From the analysed materials it follows that at the delayed sowing of cows after calving, as occurred in the case investigated by us, there is an increase in the duration of lactation. Regardless of the origin of Holstein cows the duration of lactation is well superior the limit of 270-305 days.

Despite the fact that in the Holstein population, originating in the Netherlands, the duration of lactation is 65 days longer (+21.5%), compared to the optimal-accepted duration (305 days) however it was found to be the best result compared to the other two population.

The longest duration of lactation is attested in the population of Holstein-Prim cows French origin, exceeding the maximum permissible limit by 13.4 days (+43.1%) and by 66.4 days (+17.9%) (td = 2.66; P≤0.01) originating of the Netherlands, vis-à-vis the population of German origin the difference is 35 days (8.7%) in favour of the latter.

The results established at the German Holstein cow population reveal that the duration of lactation is longer, compared with maximum permissible limit by 96.4 days (+31.6%), at the same time, it goes beyond, with a genuine difference, in this respect the Holstein population of Dutch origin by 31.4 days (td=3,78; P≤0,001.

The increase, in excess of the duration of lactation, although it follows the production of a large quantity of milk, calculated per day lactation, from these cows is obtained a smaller quantity of milk compared to cows having the normal duration of lactation. Too long lactation brings with it 15% loss of milk.

As we mentioned in the achievement of the objective one calf per cow/year and high milk production per lactation and the entire operating period depends on the duration of the service-period, as this is reflected on the interval between calving (Fedoseeva, 2007; Gabor, 2008).

In order to assess the effectiveness of the exploitation of Holstein milk cows of different origin we determined the duration of the interval between calving in the respective populations (Figure 4).

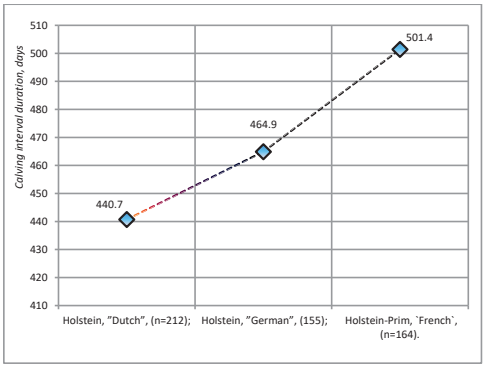

Figure 4. Duration of the interval between calvings at Holstein cows, different origin, exploited in RM

The data obtained on the value of the intreval between calvings at Holstein cow populations show that regardless of the origin of the animals it significantly exceeds the maximum allowable value of 365 days, but is differentiated by origin, forming the followin string:Holstein Dutch < Holstein German < Hostein-Prim French.

So, but in Holstein cows, Dutch origin the duration of the interval between calvings prevails the maximum allowable value by 75.7 days (+20.6%), being less by 24.9 days (-5.7%) compared to the established result at the Holstein cow population, German origin and by 60.7 days (-13.8%) (td = 2.22; P≤0.05) to cows of Holstein-Prim, French.

At cows originating from Germany the duration of the period between calvings is greater compared to the maximum accepted value by 99.9 days (+27.4%). The worst results were found to be at the Holstein-Prim cows population of French origin, where the difference is greater, with 136.4 days (+37.4%) to optimal value and with 36.5 days (+8.3%) compared to cows of german origin.

The most relevant result of the coefficient of use of the reproductive capacity of cows is at cows originating in the Netherlands, exceeding those of german origin with a genuine difference of 0,05 (td = 3.57; P≤0.005) and non-authentic of 0.06 Holstein-Prtim cows, french origin (Figure 5).

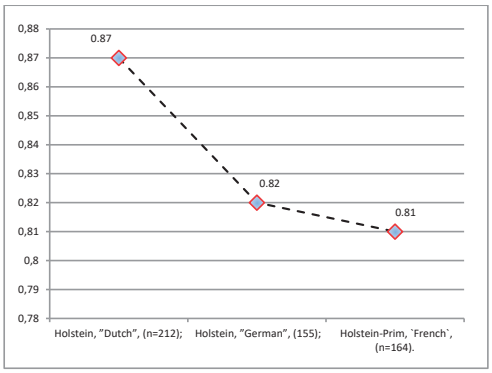

Figure 5. The coefficient of use of reproductive capacity at Holstein cows populations, different origin, exploited under RM conditions

Under the same conditions, at the last two populations the results are similar, the difference being 0.1 in favor of the German population.

In the context of the reports we note that the functionality of the reproductive apparatus is influenced by both genotype and external factors. But starting on the fact that the manifestation of the phenotype represents the interaction between genotype and environment, defined as the norm of reaction, based only on these data, we are far from determining what is the share of influence of the genotype and to what extent external factors (technological and ambient) participate.

Knowledge of the degree and direction of the correlational links between breeding indices and milk productivity at the studied cow populations is of interest because when selected characters at the cattle for milk production correlate positively, selecting the animals after high milk production, the breeding indices will evolve in the same direction.

If there are negative correlations between the desired characters, the selection work is intertwined, since the increase of one character will contribute to the shrinking of the other.The results of the interrelationships between the duration of the service-period, the interval between calvings and the milk productivity indices of Holstein cows of different origin (Table 1) highlight the presence of correlational links, both positive and negative of very different degree.

Table 1. Dynamics and direction of correlation coefficient between breeding indices and milk productivity at the Holstein cow population, different origin

Specification	Holstein, Dutch, n = 212		Holstein, German, n = 155		Holstein-Prim, French, n = 164	
	SP	CI	SP	CI	SP	CI
Duration of lactation (DL)	0.59±0.04	0.97±0.004	0.96±0.01	0.99±0.001	0.72±0.04	0.99±0.004
Milk on total lactation (MTL)	0.44±0.06	0.74±0.03	0.92±0.01	0.91±0.01	0.64±0.05	0.64±0.05
Overall fat on total lactation (OTL)	0.40±0.06	0.69±0.04	0.92±0.01	0.91±0.01	0.63±0.05	0.60±0.05
Milk on normal lactation (MNL)	0.19±0.07	0.23±0.07	0.58±0.05	0.54±0.06	0.10±0.08	0.05±0.08
Fat on normal lactation, (FNL)	-0.05±0.07	0.04±0.06	0.30±0.07	0.31±0.07	-0.04±0.08	-0.29±0.07
Overall fat on normal lactation (OFNL)	-0.02±0.07	0.17±0.07	0.58±0.05	0.54±0.06	0.12±0.08	0.04±0.08

Thus, between the duration of lactation and the breeding indices (SP, CI) there is evidence of a very high positive correlation at the population of Holstein cows of German origin, high and very high at Holstein-Prim French cows, reasonable and very high at Dutch Holstein cows.

Between the duration of the breeding indices (SP, CI) and the milk productivity indices on total lactation (milk and overall fat) there remain very high positive correlation links to Holstein, German, high to those of French origin and reasonable to high at the Dutch Holstein population.

Analyzing the results obtained on the correlation between the main indices of milk productivity on normal lactation with the duration of the SP and CI, we mention the drastic decrease in the size of the correlation coefficient.

Here we attested the presence of the reasonable positive correlation between the duration of the service-period and the interval between calving

with the quantity of milk at German Holstein cows, in the other two populations - the weak and very weak correlational links.

Regarding the interrelationships studied between the breeding indices and the average milk fat on normal lactation the size of the correlation coefficient is constantly descending, being weak positive at German Holstein population and very weak and weak reversing its direction to the Dutch Holstein cows and the French Holstein-Prim cows. The similar trend is also observed in the sky regarding the interrelationship with the overall fat on normal lactation, the links with it and the duration of the service-period and the interval between calving are reasonable positive in German Holstein cows, and very weak positive in the other two populations. T

he results obtained in the study show (Figure 6) that the coefficient of use of the reproductive capacity of cows and the indices of milk productivity (duration of lactation, milk, mean fat and overall fat) on total lactation are attested to the high and very high negative correlation.

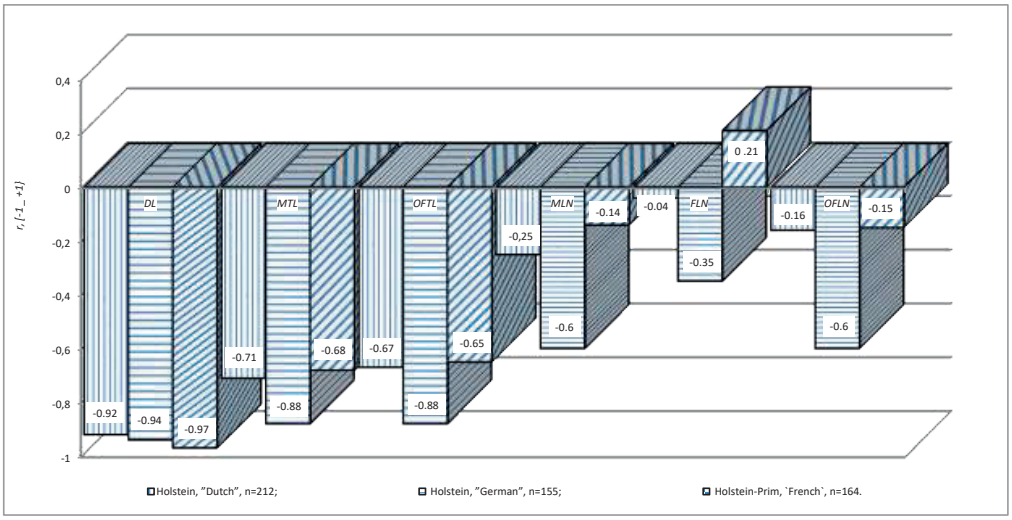

Figure 6. Interrelationships between the coefficient of use of reproductive capacity and the index of milk productivity at Holstein cow populations, different origin, exploited under the conditions of the Republic of Moldova

With regard to the main indices of milk productivity on normal lactation (milk, mean fat and overall fat) and the coefficient of use of the reproductive capacity of cows, the presence of very weak and weak negative correlation is attested, with the exception of the average fat content in milk at cows of French origin, at which the coefficient of correlation between the studied indices is positive.

The strongest negative correlation altogether link is attested between the duration of the coefficient of use of the reproductive capacity of cows and the duration of lactation, regardless of the animal's origin.

The data obtained during 3 years of monitoring of the breeding indices and their interrelationship with the milk productivity at cows of Holstein breed of different origin, now and exploited in different areas of the Republic of Moldova, once more prove, that the breeding of cows with high milk production remains problematic and needs more rigorous work to select individuals who have both characters with high values.

CONCLUSIONS

The value of the main breeding indices at Holstein cow populations, different origin exploited under the conditions of the Republic of Moldova, in the average per population, significantly exceed the maximum permitted limits.

In the Population of Holstein-Prim cows of French and German origin, the duration of dry period exceeds the maximum allowable value (60 days) by 5.6 and 5.7 days (+9.2%).

The largest gap was established at Holstein population of cows originating in the Netherlands, with the optimal value of 9 days (+15.0%), compared to populations of French and German origin the difference is, respectively 3.4 days and 3.3 days (+5.0% and +5.2%), being non-authentic.

The duration of the service-period, regardless the origin of the cows, is significantly higher compared to the maximum allowable value even for cows with very high milk production.

The best results were established at Dutch Holstein cows. At the Holstein, German cow population the service-period duration is increasing vis-à-vis premiums with an inauthentic difference of 7.1 days (+3.8%). The results obtained at Holstein-Prim, French origin, are the highest, exceeding the Holstein Dutch with an inauthentic difference of 32.9 days (+17.4%) and with 5.8 days (+13.1%) Holstein cows, German origin.

The duration of lactation in the population of Holstein cows of Dutch origin is the best result compared to the other two populations. At Holstein-Prim cows, French origin is attested the longest duration of lactation, exceeding the maximum allowable value by 131.4 days (+43.1%), by 66.4 days (+17.9%) (td = 2.66; P≤0.01) originating in the Netherlands, vis-à-vis the population of German origin, difference is 35 days (8.7%) in favour of the latter.

The results established at the German Holstein cow population the duration of lactation is higher, with a maximum permissible limit of 96.4 days (+31.6%), at the same time, exceeds, with a genuine difference, in this respect the Holstein population of Dutch origin by 31.4 days (+8.5%) (td=3.78; P≤0.001)

The results established at German Holstein cow population the duration of lactation is higher, with a maximum permissible limit of 96.4 days (+31.6%), at the same time, exceeds, with a genuine difference, in this respect the Holstein population of Dutch origin by 31.4 days (+8.5%) (td=3.78; P≤0.001).

The duration of the interval between calving, regardless of the origin of the animals, significantly exceeds the maximum allowable value, but differentiated by origin, forming the following string: Dutch Holstein (75.7 days) < Holstein, German (99.9 days) < Holstein-Prim French (136.4 days).

The most relevant results of the cow's reproductive capacity use were achieved at cows originating in the Netherlands, exceeding those of German origin with a genuine difference of 0.05 (td=3.57; P≤0.005) and non-authentic of 0.06 Holstein-Prim cows of French origin.

Between the coefficient of use of the reproductive capacity of cows at the investigated populations and the indices of milk productivity (milk, mean fat and overall fat) on total lactation was established the presence of high and very high negative correlational links and, on the other hand, negative from reasonable to weak with milk productivity indices on normal lactation (milk, mean fat and overall fat).

The highest negative correlational link (-0.94 – -1.0) is attested between the duration of lactation and the coefficient of use of the reproductive capacity of cows.

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BIOLOGICALLY ACTIVE PREPARATIONS AND REPRODUCTION INDICES IN DAIRY COWS

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Abstract

The biologically active preparation under the symbolic name "Bison" was administered to pregnant cows for a period of 150 days (60 days before calving and 90 days after calving) 20 ml/head/day. As a result of the research it was found that the cows in the experimental group had no complications in calving, the first estrus manifested after 27.3 days, each cow was seeded as a result in the first estrus, spending only 2 doses of frozen semen, while in the cows from the control group, which did not receive the preparation "Bison", the first estrus manifested after 36.2 days, each cow was inoculated 1.75 times, spending on average 3.5 doses of M.S.C. for a gestation of a cow. Fertility was 2 times lower in the control group (50%) than in the experimental group (100%). Thus, the administration of the nominated preparation, cows in the last 2 months of gestation and the first 3 months after calving, in the amount of 20 ml/head/day, led to a decrease in the number of artificial inseminations for a gestation by 0.75 units, to a decrease in semen costs by 1.5 doses/cow, and to an increase in fertility by 50%.

Key words*: cows, fertility, preparation, semen.*

INTRODUCTION

For the last period of gestation, highly productive dairy cows must be prepared not only to give birth to healthy and vigorous calves but also for milk production in the future lactation. It is also very important at the same time to maintain the health of the cow in order to achieve this productivity. At dairy cows with high productivity in the postpartum period, disorders of the reproductive system are quite common. These disorders are characterized by heat delay, repeated artificial insemination, increased length of service period, which in turn lead to higher semen costs, decreased fertility and reproductive capacity, length of calving-interval and as a result decreased amount of milk per lactation. Causes of reproductive disorders are many and varied, but it is established experimentally by several researchers, that the main cause is the so-called phenomenon of dominance of the milk production process in large quantities, which in turn inhibits to some extent other systems and processes including and the reproductive system. This dominance depends not only on the breed of cows, but also on other technological factors such as maintenance systems, methods of exploitation, etc. For the normalization of reproductive processes at cows with high milk production, animal owners use different methods and procedures, starting with the use of medicines and preparations to stimulate heat, improve maintenance conditions, improve nutrition, up to the formation of breeds and lines of animals with characteristics normal reproductive system. The selection of cows according to their reproductive characteristics is a procedure that is still practiced today in the breeding farms of some economic agents. In the research undertaken by several scientists, in terms of improving the reproductive qualities of lactating cows in the postpartum period, the methods of improving nutrition by introducing in animal rations food stimulants from the spectrum of premixes, probiotics, prebiotics, other micro- and macro elements supplements are predominant. These additives acting as nutritional stimulants, concomitant also act as stimulators of other metabolic processes, including animal reproductive processes, thus

influencing the natural resistance of the body as a whole. At the same time, we mention the fact that the influence of these preparations on the metabolic, energetic processes, of the reproductive functions of the highly productive cows, requires in-depth and detailed studies that remain still current (Misailav, 1976; Mihailov, 1976; Șacalov, 1956).

Approaching this topic, through the prism of abundant nutrition, and trying to balance and ensure the ration with the full range of nutrients in the postpartum period is a desideratum of the purpose of diminishing the reproductive problems of the highly productive dairy cows during this period.

An imperative significance has the composition and origin of the preparations used as stimulators of the metabolic processes in the body of cows (Rusakov, 2015; Yarmots et al., 2019). In order to improve the reproductive indices of dairy cows, we initiated an experimental research based on the use in animal feed of biologically active preparations synthesized from the residues of the beer production industry. The experimentally active biological preparation presents an extract of natural products, a concentrate of amino acids, micro- and macroelements, enzymes, ferments, other biologically active substances, which should, beneficially influence the reproductive processes of high-yielding dairy cows both during pregnancy and in the postpartum period (Sinelschikova et al., 2020).

MATERIALS AND METHODS

For the development of experimental research, 2 groups of cows with 5 heads in each group were selected. The cows at the beginning of the research were in the 7th month of gestation and the experiments were extended after calving for another 3 months, so the total experimentation period lasted 150 days. During this period, the cows from the experimental group were administered the biologically active preparation "Bison", in liquid form, in an amount of 20 ml/ head/day. This dose of the preparation was included in the ration once a day during the administration of the combined fodder. Both groups of animals (control and experimental) received identical rations throughout the experiment. During the research, the course of calving, the absence or presence of complications at calving and after were monitored, and some reproductive indices that appeared at animals in the postpartum period were further calculated and analyzed. Thus, the period of manifestation of the first estrus, the duration of the service period, the number of artificial inseminations performed until obtaining the gestation, the conception rate, the expenses of semen for a fertile insemination, etc. were established. In order to establish the influence of the biologically active preparation on the blood indices, blood samples were taken from all cows included in the research. Subsequently, the assessment and analysis of blood indices were performed, which characterize some changes and modifications in the metabolism of the cow's body during the experiments. Urea, triglycerides, glucose, amylase, total protein, albumin, alkaline phosphatase (ALP), creatinine, calcium, phosphorus and magnesium were studied in blood serum samples using the StatFAX semi-automatic biochemical analyzer with special ELITech putties.

RESULTS AND DISCUSSIONS

A calf must be obtained from each cow in a calendar year, so that the interval between two consecutive calvings "*calving-interval*" must not exceed 12 months. To obtain this result, the cow must be sown in the first 3 months after calving. This result can be obtained only if the calving proceeds normally, the reproductive organs become involved and return to normal in a short period, and the first estrous cycle occurs 25-35 days after calving. Subsequently, when the cow for various reasons is not sown in the first estrus, the estrous cycles are repeated regularly at intervals of 18-21 days. Experimental research began in the last 2 months of gestation to monitor the effects of the biologically active preparation on the course of calving, the manifestation of estrous cycles and the results of artificial insemination in the postpartum period. After completing the experimental research, the data obtained were processed and analyzed to assess the effects obtained. At cows from the experimental group, complications during gestation were not detected. At the end of gestation, it was found

that the calving of the cows in the experimental group took place normally, without any complications or help from the veterinarian. Were born healthy, viable calves that were later included in the technological process of raising young. For the last 2 months of gestation in the control group were observed deviations from the normal behavior and health of a single cow, which was expressed by the restlessness of the cow, decreased appetite, looking for a place more separate from other cows. Later, this cow had serious complications during calving and was rejected for this reason. In 4 other cows of this group, the last 2 months of gestation went normally, there were no complications during calving and after. After calving, at cows with high milk production, all the body's metabolisms are mainly directed towards fulfilling a single goal: stimulating the milk production processes. In most cases, the activation of these processes is detrimental to other functions that are quite vital during this period, such as the involution of the reproductive organs and their return to normal. Because of this, is slowed down this recovery and the period of manifestation of the first estrus is extended. The manifestation of estrus in the postpartum period at cows of the experimental lot was after 27.3 days after calving, and in the control group after 36.2 days. The duration of the service period was by 8.9 days longer at cows in the control group than at those in the experimental group. Concidering that the first estrus at cows involved in research manifested itself in a fairly short time after calving, when the reproductive organs have not yet returned to normal, the cows in both lots in this intentional estrus were not inseminated. Until the manifestation of the next estrus, at most cows the reproductive organs returned to normal and were prepared for artificial insemination.

In the second estrous cycle each cow in the experimental group was inseminated as a result only once. All cows in the control group were artificially inseminated in the 2nd estrus, and three of them were repeatedly inoculated in the 3rd estrus. Thus, the number of inoculations of cows in the control group was higher due to the repeated inoculation of 3 cows in the third estrous cycle. As a result, in the experimental lot 4 inseminations (4 cows) were performed, one insemination per cow, and in the control lot 7 inseminations (4 cows) 1.75 inseminations per cow (Table 1).

Table 1. Reproductive indices of cows

No	Indices	Control group	Experimental group	+,- by control
1	Duration of service period, days	36.2	27.3	-8.9
2	Insemination for a gestation	1.75	1.0	-0.75
3	Fertility, %	50.0	100.0	+50.0

Two doses of frozen semen are spent on each artificial insemination of the cows. Because in the control group several inoculations were performed on one cow, or several doses of semen were spent for the artificial insemination of a cow - 3.5 doses, while in the experimental lot where each cow was inoculated only one given, were spent only 2 doses. Fertility was by 2 times lower in the control group (50%) than in the experimental group (100%), considering that the cows in the experimental group became impregnate after the first insemination, and some cows in the control lot became impregnate after the second insemination.

As a result of experimental research on the study of the new biologically active preparation, synthesized by researchers from the Institute of Microbiology and Biotechnology in brewer's yeast, it can be said that the nominated preparation used in feeding of impregnate cows positively influences some aspects of reproductive indices in the postpartum period. Thus, the administration of the nominated preparation to cows in the last 2 months of gestation and the first 3 months after calving, in the amount of 20 ml/head/day, leads to a decrease in the number of artificial insemination by 0.75 units/cow, reduces semen costs by 1.5 doses/cow, and increases fertility by 50%. During the experiment, in order to have a broader picture of the metabolic processes carried out during this time, blood samples were taken from the cows in the study.

Blood plays a very important role in the body of animals, ensuring the exchange of substances in the cells and tissues of all organs. Blood biochemical indices characterize metabolic processes as disturbances of these processes influence blood indices (Gromyko, 2005; Kazartsev, 1986; Danilov, 2008)

The metabolic processes of the animal organism are reflected on blood indices that

change quantitatively, depending on their physiological state, and can characterize the situation in which the animal is at the moment. In order to detect the changes of some blood indices that may occur following the administration of the biologically tested active preparation, blood samples were taken and analyzed from all cows in the research. Blood samples were taken at the beginning and end of the experiment, the blood indices obtained are presented in Tables 2 and 3.

Blood indices throughout the research in both lots were within the normative limits, regardless of the physiological stage in which they were. Following the analysis of blood indices at the end of the experiment, quantitative differences were found between the experimental and control groups in most of the analyzed substances. These differences are explained by the fact that the cows at the end of the research were in another physiological stage, namely at the top of the lactation curve.

Table 2. Blood indices of cows at the beginning of the experiment

Indices	Group	
	Experimental	Control
Ca, mmol/l	12.8±2.3	16.7±3.3
P, mmol/l	2.9±0.4	2.8±0.4
Mg, mmol/l	2.5±0.5	3.4±0.6
Protein, g/l	50.8±6.9	44.3±3.8
Albumin, g/l	19.9±2.9	29.2±1.6
Creatinine, mmol/l	104.1±25.2	101.8±29.8
Urea, mmol/l	5.5±0.9	8.3±1.0
Glucose, mmol/l	5.4±0.8	4.2±0.5
Triglyceride, mmol/l	1.3±0.4	0.7±0.1
Cholesterol, mmol/l	172.5±69.0	90.0±9.7
Amylase, u/l	165.5±17.8	129.8±19.3
Alkaline phosphatase, u/l	64.0±17.7	67.3±24.3
Fe, mkmol/l	0.8±0.1	0.9±0.1

Table 3. Blood indices of cows at the end of the experiment

Indices	Group	
	Experimental	Control
Ca, mmol/l	8.2±0.5	13.0±2.00
P, mmol/l	0.6±0.1	0.3±0.04
Mg, mmol/l	0.9±0.2	1.4±0.60
Protein, g/l	12.3±2.2	15.9±2.82
Albumin, g/l	11.0±2.3	8.4±0.96
Creatinine, mmol/l	36.4±25.7	48.8±13.71
Urea, mmol/l	0.3±0.0	0.4±0.04
Glucose, mmol/l	3.2±0.1	3.0±0.25
Triglyceride, mmol/l	3.1±0.1	3.3±0.14
Cholesterol, mmol/l	380.4±22.5	378.7±20.08
Amylase, u/l	11.9 ±3.8	10.8±2.81
Alkaline phosphatase, u/l	6.4±1.5	13.4±3.35
Fe, mkmol/l	3.6±1.2	3.7±0.48

Thus, at the end of the experiment, at the cows from the experimental group there was a decrease in the level of protein, albumin, urea, glucose, Ca, P, Mg, amylase, alkaline phosphatase, compared to those in the control group.

The conglomerate of listed substances, participates intensively in milk production, by intensifying the metabolic processes to ensure the vital functions of all organs including those involved in milk synthesis. The amount of protein in the blood is an important indicator of metabolism in the body of the cow being the fact, that proteins ensure the functioning of all vital and productive organs of the animal. In the case of our experience, the decrease in protein levels from 50.8 g/l to 12.3 g/l in the blood indicates the rapid increase in milk production, which was found during this period. The final products of protein breakdown, urea (from 5.5 - to 0.3 mm/l) and creatinine (from 104.1 - to 36.4 mm/l) also decreased.

An increase of the level of triglycerides in both lots of cows in the postpartum period indicates an increase and accumulation of energy in the body. This can be qualified as a preparation of cows for the next physiological period, ie for the activation of the reproduction processes and the installation of the next gestation. Also, this quantitative increase in triglycerides contributes to some extent to an improvement in the immune system, which in turn ensures good health for cows. The elements calcium and phosphorus in the postpartum period, decreased in both groups due to the elimination from the body through the produced milk, remaining at the same time, as mentioned above, within the normative limits.

The results show that the biologically active preparation "Bison", included in the ration of pregnant cows in the last 2 months of gestation and the first 3 months after lactation, had a positive influence on many metabolic processes of the body. The results regarding the improvement of reproductive indices, the birth of healthy and viable calves, the increase of milk production, are visible and in our opinion, it is largely due to the biologically active preparation administered. In this context we see the need to analyze the content of the biologically active preparation experienced, in

order to explain some extent of this influence, on what factors it is based and how it acted. The detailed knowledge of the content of the preparation, the number and quantity of biologically active substances in the preparation, the quantitative proportions between substances, helped us to support our assumptions regarding the mechanism of influence. The latest research in human and animal physiology, both fundamental and applied, focus attention to the fact that the interaction between conglomerates of nutrients, micro and macroelements, vitamins, enzymes, hormones, ferments, etc., occur in certain periods of time and only with meeting all the beneficial factors. The lack of only one element of this chain at a certain time, can stop the previously triggered metabolic process, or the process can be directed elsewhere, sometimes with serious unfavorable consequences for the body. Given the fact that metabolic processes in the animal body are a component of the whole system functioning, regulation and ensuring the vitality of the animal, the inclusion of a preparation or nutrients in the ration of animals should be studied and analyzed only in complex with the whole system and reciprocal interactions of various processes. Of course, we are aware that in our rather small and limited conditions and capacities, it is almost impossible for such experimental research to study the integral vital system of the animal, and for this reason we have to limit ourselves to studying a very narrow segment of the process. metabolic food.

In many scientific papers (Bestujev, 1963; Schmidt, 1957; Oldham, 1987) the influence of only one element is studied, for example selenium or iodine, excluding somehow or placing in the background the influence of other substances that interact with the experienced elements. Of course, it is very difficult to find, select, synthesize such a nutrient or food additive that contains a conglomerate of elements that fully meet the requirements of the animal body, ensuring the normal functioning of all metabolic processes and vital organs. It is even more difficult to look for and determine such a nutrient that is of natural origin, with biologically active properties, without toxic ingredients, and without adverse influences.

Researchers from the Microbiology and Biotechnology Institute of the Republic of Moldova conducted studies and experimental research to search for, identify and select such a nutrient or food additive that is of natural origin, contains many valuable nutrients and is harmless. Studying several raw materials, including residues from industrial processing of crops, or identified products from the wine processing and brewing industry. As a result of the fermentation processes, wine and beer yeasts are obtained, which also served as raw material for the synthesis of the new biologically active preparation, under the conventional name "Bison", which was later studied, experimented and analyzed in experimental animal research. by researchers from the Scientific and Practical Institute of Biotechnologies in Zootechny and Veterinary Medicine.

The nominated preparation was obtained by extraction from yeast biomass of biologically active preparations of amino acid-protein, polysaccharide and lipid nature, and subsequently, following laboratory analyzes, it was established that this preparation contains a wide range of nutrients, proteins, carbohydrates, lipids, fatty acids, micro and macroelements, a wide variety of amino acids, a series of micro and macroelements, etc. This biologically active preparation synthesized from the residues of the food industry, with such a chemical composition of precious substances, experienced in our research on cows with high milk production, has led to quite positive results in improving reproductive indices.

In this context, we can assume that the experienced preparation contributes to some extent to ensuring the normal functioning of many vital organs of the animal body. The substances and elements in this preparation probably participate in the closely interconnected complex reactions that take place in the body, through the influence of nutritional factors on animal health. Perhaps this multitude of elements, some of which are found even in a smaller amount, can participate as catalysts, stimulators and triggers of other reactions and vital processes, as a result of which such substances are synthesized that are not even found in food, or in other preparations

administered to animals in various forms, but which are very necessary to ensure the health of the animal.

CONCLUSIONS

The biologically active preparation "Bison" administered to dairy cows positively influences reproductive indices in the postpartum period: the manifestation period of the first estrous cycle is reduced; decreases the number of artificial inseminations to obtain gestation by 0.75 units/cow; reduces semen costs by 1.5 doses/cow; increases fertility by 50%.

The parameters of the blood indices undergo non-essential changes between the groups of animals following the administration of the preparation "Bison"

The biologically active preparation "Bison" administered to dairy cows contributes to increasing the immune resistance of animals and as a result to maintaining their health.

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STUDY ON QUANTITATIVE INDICATORS FOR RAW SKIN OF MALE CALVES OF BEEF BREEDS

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Abstract

The changes made in the composition and structure of beef cattle breeding in Bulgaria, impose along with research in the field of nutrition, technology and meat productivity to study the skin as a raw material for various branches of light industry. The relative weight, the percentage of the living mass of skin, the sizes, the thickness of skin in different areas were studied and skin area in fattened male Simmental, Limousine, Hereford and Aberdeen Angus calves slaughtered at 15 months of age. There are significant differences in the studied quality indicators of raw skin. The highest is the relative weight of the raw skin of calves of the Simmental breed, which showed the heaviest 38.9 kg and thick skin-6.94 mm at point "O" and 6.71mm at point "H".

Key words*: area, beef breeds, calves, skin, weight.*

INTRODUCTION

The changes made in cattle breed composition in Bulgaria in recent years, suggest not only to study meat productivity and growth ability but also to conduct studies on the skin as a raw material. Raw cattle skin is a major component of the footwear, leather, fur and haberdashery industries (Balabanov, 1975; Gaidar, 2010; Kibkalo et al., 2014; Gergovska and Panayotova, 2016).

The skin is associated with fundamental vital functions in the body: protective, regulatory and excretory. The condition and shine of the hairy cover give information about the general health of the cattle. The skin makes up about 7-9% of the animal's live weight (Sinivirski & Petkov, 1986; Hamid et al., 2000; Kibkalo and Gersbilov, 2009; Badahov, 2011; Panin, 2015; Belkov and Panin, 2017).

Beef breeds show a large live weight and heavy and dense skin, as a rule they reach about 35-40 kg, relative weight and thickness in the controlled areas from 5.6 to 7 mm (Badahov, 2011; Irgashev & Kosilov, 2014; Kibkalo et al., 2014; Kozyr, 2018; Lonegau et al., 2019).

Weight, surface area, smoothness and skin defects in cattle depend on the species, genetic capabilities of the breed, sex, age and live weight, as well as thickness and density, containing different amounts of moisture, salts and aggravators, as well as feeding, technology and the environment (Maddox & Jakson, 1988; Besedov, 2007; Herring, 2014; Nezavitin et al., 2015; Adzinova & Mambetov, 2018; Popsuy et al., 2020). The objective of the present study was to examine the quantitative indicators: weight, percentage of live weight, size, surface area in dm² and thickness of raw skin in certain body sections of Simmental, Limousine, Hereford and Aberdeen Angus beef calves slaughtered at 15 months of age.

MATERIALS AND METHODS

This is the fourth paragraph from Materials and Methods that should be replaced with your content. It only contains example text and proper formatting. The experiment was conducted with calves from the farm of the Experimental Base of RIMSA-Troyan and small farms from the region in 2019 and the winter of 2020. The objective of the study were male purebred calves, which were fattened up to 15 months of age. Raw skins were studied of slaughtered animals of breed, such as: Simmental, Limousine, Hereford and Aberdeen Angus with different live weight, 10 raw skins of male calves per breed. The animals were kept under the same conditions, free-boxing

and fed traditionally. The slaughter and skinning took place in Meat Factory - Troyan. The skins were removed in two stages: by manual skinning of individual areas and final mechanical skinning.

Instrumental methods and technical measuring instruments were used. Live weight was determined by weighing with an electronic scale to the nearest 0.01 kg. The carcass weight of the calves and the relative weight of the raw skins were determined immediately after slaughter and cleaning from aggravators using an electronic scale accurate to 0.01 kg. The dimensions were taken by the help of a standard roulette.

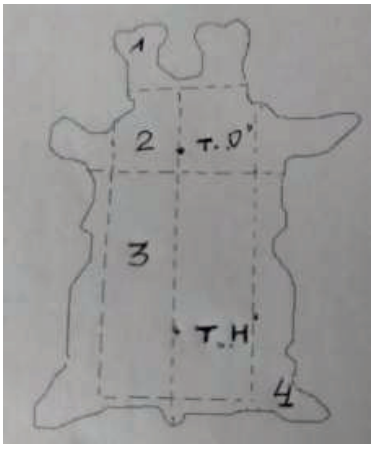

Figure 1. Topographic characteristics of fresh bull skin: 1. Head; 2. Nesk; 3. Central part; 4. Read part; 5. t "O"; 6. t. "H"

The area of the skin was determined by the method of total squares in dm², according to a scheme, using a wooden meter divided into dm, and the thickness with a caliper at two specific points: at a standard point "O" in the area of the withers and a standard point "H" in the skin area of the sacrum according to the method of Arzumanyan with an accuracy of 0.1 mm (1962).

The data were processed by the methods of variation statistics with the help of "Statistica-2000" program and presented in tables.

RESULTS AND DISCUSSIONS

Raw skin is the skin removed from the carcass of an animal after slaughter and skinning. Used mainly as a term for operating production data. There is no globally accepted unit of measurement for the production, marketing or use of skin data. Data are usually presented differently in statistical information in terms of number or weight, while information on leather products is presented in terms of surface area or weight (FAO, 1994).

The skins of different cattle breeds differ in their structure and technological qualities. The weight of raw skin depends primarily on its size, thickness and density (Adzimova & Mambetov, 2018).

The relative weight, percentage of live weight, size, area and thickness of raw skin in certain areas of fattened calves of the studied breeds are shown in Table 1.

Table 1. Indicators of raw skins of fattened, meat-producing calves slaughtered at 15 months of age (X ± Sx)

Breed ♂	n	Qualitative indicators of raw skin						
		Skin weight		Length cm	Width cm	Surface area, dm²	Thickness at point "O", mm	Thickness at point "H", mm
		kg	% of live weight					
Simmental	10	38.90*±1.03	8.69	180.72±1.73	194.53±2.47	369.29±6.88	6.94**±0.26	6.71**±0.30
Limousin	10	36.24*±1.31	8.27	174.17±3.71	188.74±2.05	347.46±10.66	6.48**±0.23	5.91**±0.17
Hereford	10	34.48*±1.19	8.07	165.33±2.17	176.08±2.43	299.57±22.28	6.17*±0.35	5.65**±0.25
Aberdeen-Angus	10	30.44*±1.21	7.48	161.24±1.44	171.94±2.27	285.23±11.24	5.17**±0.23	4.86**±0.19

*P<0.05, P<0.001**, P<0.001***

The skin weight is determined by the structure of the collagen fibers. Raw skins obtained from cattle are subdivided into light skins weighing up to 25 kg and heavy skins weighing from 26 to 60 kg. The technological cycle with a duration of 450 days allowed us to get heavy skins with a relative weight of 30.44-38.90 kg.

The relative skin weight was measured immediately after skinning and removing dirt and manure from it. Significant differences in the relative skin weight of the studied male calves were observed. Simmental breed showed heavier raw skin by 8.46 kg compared to the Aberdeen Angus breed, by 4.42 kg compared to Hereford breed and by 2.66 kg compared to Limousine breed, or 21.7% respectively, 11.36% and 6.84% more ($P<0.05$). It is noteworthy that at 15 months of age the raw skins of experimental animals are heavy and meet the requirements for relative weight (Sinivirski and Petkov, 1985; Kibkalo et al., 2014).

By percentage of raw skins in relation to animal weight, the ranking is led by the raw skins of Simmental calves with 8.69%, followed by those of the Limousine breed with 8.27% and those of Hereford breed with 8.07%. The lowest in value, but within the normal range are the results obtained by calves of Aberdeen Angus breed 7.48%.

The skin size is an important indicator of its technological qualities. The highest values are measured in Simmental - length 180.72 cm and width 194.53 cm, and the lowest values in Aberdeen Angus - length 161.24 cm and width 171.94 cm, or differences of 19.48 cm for length and 22.59 cm for width.

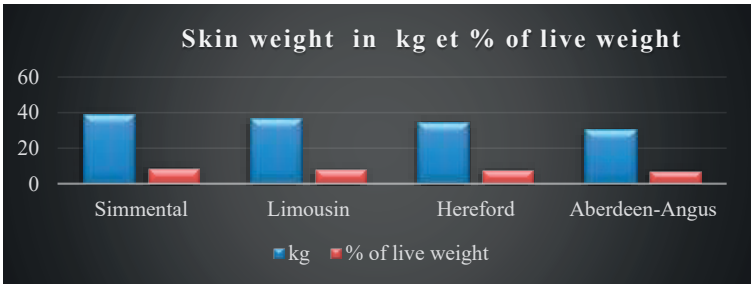

Figure 2. Skin weight (kg and % of live weight)

The surface area of the skin is an indicator for determining its value. Raw skins obtained from Simmental breed had the best spatial spreading - 369.29 dm², followed by the Limousine breed - 347.46 dm², Hereford breed - 299.57 dm² and Aberdeen Angus breed - 285.23 dm², or differences of 21.77 dm², 69.72 dm² and 84.06 dm² ($P<0.05$). Interbreed differences in this indicator are insignificant ($P<0.05$).

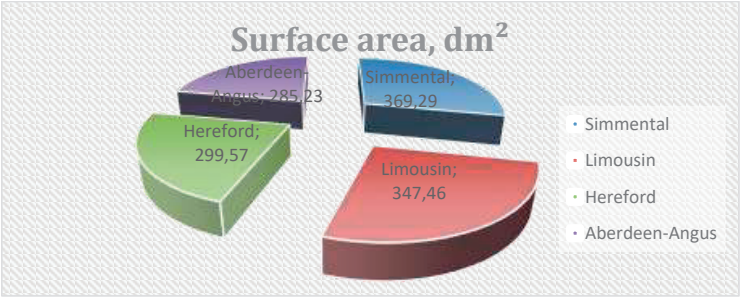

Figure 3. Surface area, dm²

The skin thickness is different and depends on the type, breed, sex, season, as well as the topographic area where the measurement was performed. The thickest skin among the examined raw skins in the specific, studied standard areas (point "O" and point "H") were registered in Simmental 6.94 mm and 6.71 mm, followed by Limousine, respectively 6.48 mm and 5.91 mm, followed by the representatives of Hereford breed 6.17 mm and 5.65 mm. The thinnest skins were measured in Aberdeen Angus animals with 5.17 mm and 4.86 mm. The obtained results are reliable at ($P<0.01$).

Table 2. Live and slaughter weight, ratio of 1 dm² skin/1 kg live weight, weight of 1 dm² and reduction of skin area in the transverse and longitudinal direction in % of the examined calves slaughtered at 15 months of age

Breed ♂	Indicators				
	Live weight, kg	Slaughter weight, kg	Ratio 1 dm² skin / 1 kg live weight	Weight of 1 dm² skin, g	Reduction of skin area in the transverse and longitudinal direction, %
Simmental	457±0.49	297.05±0.55	0.81	105.34	6.9
Limousin	438±0.90	293.46±0.98	0.79	104.30	7.0
Hereford	417±0.52	262.71±0.88	0.72	115.09	6.7
Aberdeen Angus	407±0.49	248.2±0.78	0.70	106.72	6.5

P<0.05

The highest live and slaughter weight values were registered for Simmental breed, 457 kg and 297.05 kg, respectively, while the lowest values for Aberdeen Angus breed, 407 kg and 248.2 kg, respectively (P<0.05). The analyzed skin ratios in dm² to live weight show the same racial superiority.

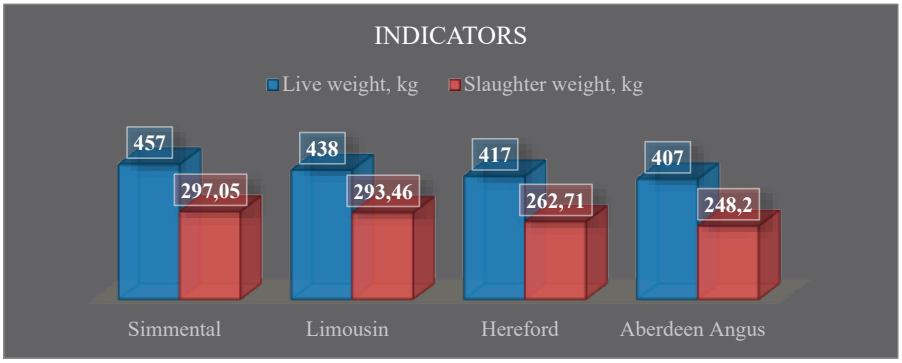

Figure 4. Indicators Live and slaughter weight calves slaughtered at 15 months of age

The highest weight per 1 dm² of skin in g was measured in Hereford breed 115.09 g, while the lowest values were registered in Limousine breed - 104.30 g. With a decrease in the skin area in the transverse and longitudinal direction in %, the values obtained are relatively close, as the skins of Limousine breed showed the best results - 7.0%.

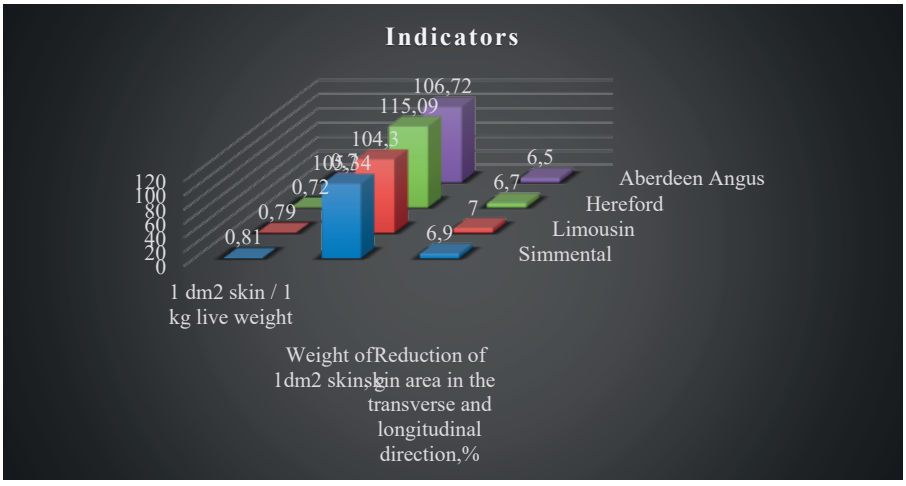

Figure 5. Indicators ratio of 1 dm² skin/1 kg live weight, weight of 1 dm² and reduction of skin area in the transverse and longitudinal direction in % of the examined calves slaughtered at 15 months of age

The data obtained in the present study correspond to the results obtained by Kibkalo et al. (2014), Panin (2015), Lonegau et al. (2019), Popsuy et al. (2020), and in some cases complement them.

CONCLUSIONS

Simmental calves showed heavier raw skin by 8.46 kg compared to Aberdeen Angus calves, with 4.42 kg compared to Hereford calves and 2.66 kg compared to Limousine calves, or 21.75%, 11.36% and 6.84% more ($P<0.05$). The thickest skins in the defined standard areas (point "O" and point "H") were measured in male animals of Simmental 6.94 mm and 6.71 mm, followed by Limousine breed, respectively 6.48 mm and 5.91 mm, followed by the representatives of Hereford breed 6.17 mm and 5.65 mm and the thinnest skins were measured in animals of Aberdeen Angus breed with 5.17 mm and 4.86 mm. The obtained results are reliable at ($P<0.05$). The analyzed skin ratios in dm^2 to live weight show the same racial characteristics. The best values for weight per 1 dm^2 of skin in g were measured in the representatives of Hereford breed 115.09 g, while the lowest values were registered in the representatives of Limousine breed - 104.30 g. With a decrease in the skin area in transverse and longitudinal direction in %, the values obtained are relatively close, as the best results were obtained from Limousine breed with 7.0%.

The duration of the technological cycle of 450 days showed an increase in carcass weight, weight and skin area among the studied animals. Calves of beef cattle raised and fed under the same conditions, give raw skins differing in quantitative indicators.

ACKNOWLEDGEMENTS

To the managements of RIMSA-Troyan and Meat Factory-Troyan for the support and assistance in the research.

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ROMANIAN RED BIOTECHNOLOGY - BLENDING TRADITION WITH STATE OF THE ART IN THE EUROPEAN AND INTERNATIONAL FRAMEWORK

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Abstract

The biotechnology field has grown rapidly in recent years. Much attention is given to the potential of the biotechnology industry, from drugs and medical devices to environmental products which have the potential to generate tremendous opportunities for society, by improving the quality of health care and producing a cleaner environment. Red-biotechnology, or medical biotechnology, is one of the branches of the biotechnology and utilizes the organism to improve health, both in the pharmaceutical and medical sectors, mainly bringing all the biotechnology applications to medicine. Romanian potential was recognized by important biotech companies as an attractive destination for biotechnology research development. Currently running large programs for research, development, and innovation, regulated within a national implementation strategy, and with an attractive higher education offer, Romania is contributing to biotech advances and has great potential for development of biological pharmaceutical products. It also represents a valuable and promising partner for future international collaborations as biotechnology continues to evolve and will remain a major field of innovation and development in many areas of the world.

Key words*: biological drugs, biotechnology industry, biotechnology regulations, national strategies, red biotechnology.*

INTRODUCTION

According to the rainbow code of biotechnology, red-biotechnology, or medical biotechnology, is one of the branches of the modern biotechnology field and utilizes the organism to improve health. Biotechnology is commonly used in the pharmaceutical and medical sectors such as in gene therapies, molecular diagnostic techniques, genetic engineering, drug and vaccine development, cancer research, regenerative therapies, biomedical 3-D-printing, veterinary biochips, cell and tissue research and assessment, stem cell research, protéomics, pharmacogenetics. The medical biotechnology field drives meetings, conferences, and workshops with participants from a variety of science, education, industry, administration, and social work fields. Biotechnology has recently become an essential component of life, in all of its aspects, but most of all in the medical area. The current interest and the magnitude of the employed research forces and economy drive are reflected by the market value in this field. It is currently estimated that the Global Red Biotechnology Market may reach 500 billion US dollars by 2026 (Acumen Research and Consulting, 2019).

EARLY BEGINNING OF RED BIOTECHNOLOGY

Since "the need is the mother of all inventions", the field of biotechnology is not an exception, as its beginning is linked to the domestication of animals, and the production and preservation of food. With the extension of food preservation methods, processed products have emerged and human diet was diversified. Cheese can thus be considered one of the first products of biotechnology, being followed by bread, vinegar, alcohol. On the other hand, in order to benefit more from domestic animals,

the cross-breeding method has been developed, the oldest example being the mule (Verma, 2011). This period, before the XIXth century is known as *"Ancient biotechnology"*.

After 1800, the next phase of biotechnology evolution, known as the *"Classical biotechnology"*, was a period of scientific blossom, starting with the heart of the biotechnology discovery, the transfer of genetic information, detailed by Mendel's "Inheritance Laws". Molecular biology started with the discovery of the nucleus, by R. Brown and nuclein, by F. Miescher. For over a century, the medical and pharmaceutical industry has been driven by technological innovation. T.H. Morgan gave us the chromosomes and the gene knowledge, while in the early XXth century, Johannsen defined the genotype and phenotype concepts. Soon after, Flemming discovered the antibiotics.

The field of biotechnology has gained a lot of momentum during the so-called *"Modern Biotechnology"* stage, with Francis Crick and James Watson's discovery in 1953 of the DNA structure, soon following the development and implementation of genetics in other fields. Further deep understanding of the gene structure was brought by Jacob and Monod, who introduced the new operon concept, while Kohler and Milestein revolutionized diagnostic techniques when they discovered monoclonal antibodies. Later on, the DNA was artificially synthesized, amplified and the first animal clone was successfully produced (Verma, 2011). Relatively recently, in 2003, The Human Genome Project was accomplished.

THE "GOLDEN AGE" OF MEDICAL BIOTECHNOLOGY

The latest progress and significant advances in Red biotechnology triggered even more research, in a circle which drove enormous financial investments, so scientists have considered this field to be the top global economic growth opportunity (Gartland, 2013). Biotechnology scope has grown to previously unimagined magnitude, and two of the most prestigious scientific journals - *Current Research in Biotechnology* and *Current Opinion in Biotechnology* - reveal the fields which are currently addressed by this multidisciplinary science as analytical, environmental, energy, chemical, plant and animal, medical, pharmaceutical, and food biotechnology, genetic and molecular engineering, and nanobiotechnology. More than anything else, biotechnology is a versatile research field, with broad and expanding range of topics.

STATE OF THE ART IN GLOBAL RED BIOTECHNOLOGY

Current scientific interests and trends. As technological innovations come from scientific breakthroughs, the heartbeat of biotechnology also lies in the research conducted at a global level. In order to identify the most prominent research themes and the major contributors on a global scale, Yeung AWK et al. (2019) managed to identify 12351 publications that were published after 2017, from more than 8500 research organizations all over the world. The authors revealed that between 2017 and 2019, the top 5 most productive countries were the United States of America, China, Germany, Brazil and India. These countries were leaders among over 140 countries/regions which were identified to have contributed to biotechnology research literature (Yeung, 2019).

Another recent study (Streltsova, 2018), reviewing the strategies and dynamics in biotechnology patenting in Brazil, Russia, India and South Africa, grouped under the acronym BRICS and commonly referred to as the "BRICS countries", is pointing out that these countries account for 25% of global biotechnology patents. The authors of this study are stressing out the fact that the capacity and the input of these significant contributors might significantly shape the current trends and the future of the biotechnology field.

Graciano et al. (2019) retrieved data from some of the most relevant Patents Databases, such as INPI, USPTO, Esp@cenet, and WIPO, and revealed that the latest Red Biotechnology Patent Applications are mainly related with cancer research, diagnosis kits, vaccines, stem cells and therapeutic antibodies. Analyzed data indicated the USA being the world leader in terms of patent application number (Graciano, 2019). In terms of scientific publications, Yeung et al. (2019) indicate that *Journal of*

Bioscience and Bioengineering was the leading journal in terms of publications metrics, followed by *Biotechnology and Bioengineering*, *Applied Microbiology and Biotechnology, Biotechnology Progress* and *Scientific Reports*. According to Yeung et al. (2019), the most prevalent biotechnology research theme was Metabolic engineering, followed by biotechnology studies involving *E. coli* and *Saccharomyces cerevisiae* about the biosynthesis of various biomolecules, such as myo-inositol (vitamin B8), monoterpenes, adipic acid, astaxanthin, and ethanol (Yeung, 2019). "Nanoparticles and nanotechnology" was highlighted as a greatly significant emerging biotechnology research field. To reveal the most prevailing biotechnology research themes of 2019, the authors analyzed the most prevalent key words in the scientific articles and revealed the top five key words as "protein engineering", "thermostability", "biofuels", "innovative biotechnologies", and "drug delivery".

Expert opinion on biotechnology risks versus socio-political factors. Scientific discoveries and biotechnology innovations have always been subjected to initial public reluctances. Although regulatory authorities, policy makers, the industry, and the general public, all have an impact on the acceptance of new trends, the scientists advocate that too little emphasis is put on the scientific principles which reflect and govern the risks and benefits behind new biotechnologies. In a very recent paper, Lassoued et al. (2019) draws attention to the fact that wide-ranging discoveries and innovation have some degree of uncertainty, which must be addressed and managed by both regulators and the industry, by responsible risk assessment. The study focuses on the contemporary and controversial subject of genome editing and discusses the probabilistic risks, hypothetical risks, and speculative risks which affect product safety and consumer perception. Lassoued et al. (2019) reveal that various countries worldwide have different approaches to risk consideration in regulating new technologies, following either a precautionary approach, or substantial evidence approach. The substantial evidence approach or scientific rationality is dictated by scientific risk assessment, while the precautionary approach is governed by other factors in the final judgement. A relevant example involved the contrasting difference between the EU and the USA in consideration and regulation of new technologies based on contrasting risk approach. While the EU is using the precautionary approach, waiting for evidence of no risks before approval, the USA approves for new technologies in the absence of verifiable scientifically assessed risk. Roberts (2018) has recently stated that the sensible approach is the science-based one, which is currently supported by scientific researchers worldwide and best reflected by the 129 Nobel Scientists campaign aiming to explain and to bring their arguments in favour of the controversial biotechnology technique of genome editing. An important alarm signal is triggered by Lassoued et al. (2019), by raising the concern that valuable advances in genome editing technology in particular and biotechnology in general, are unfortunately, currently limited by socio-political factors, which defy scientific principles.

Hurdles in global red biotechnology advances. Most surveys and reports, which point out limitations in red biotechnology development, indicate that the main hurdle is the lack of financial support for research activities and application of pilot projects. Scientific literature provides various examples, such as the National System of Innovation in Biotechnology in Brazil, which is considered only partially developed, due to deficiencies in technological advances, despite being supported by a sound and powerful scientific scope (Gabardo, 2015).

EUROPEAN STATE OF THE ART IN RED BIOTECHNOLOGY

The beginning of the modern European biotechnology era links back to 1975, three senior scientific officers of the Research Directorate General of the European Commission (Dreux de Nettancourt, Andre´ Goffeau and Fernand van Hoeck) forwarded a historical report on potentials of modern biology. Currently, as key representatives of biotechnology industry are seeking new scientific ideas, knowledge, and results, governments need to provide the right

standard of regulation. The EU is now running projects like *Horizon 2020* and the *Bioeconomy Strategy and Action Plan for Europe,* with important budgets, aiming to help create and maintain an appropriate climate for scientists and industry to establish collaboration through innovative projects and entrepreneurship. According to a report on biotechnology in Europe, "The Tax, Finance and Regulatory Framework and Global Policy Comparison", a joint report by EY and EuropaBio (Report on Biotechnology in Europe), the recent period has brought significant increase in global health care biotechnology sales and the job market.

The legislative framework remains the most significant area which must be improved in order to allow the red biotechnology market to grow. The best example would be the former Directive 2001/20/ EC on Clinical Trials of the EU, which led to difficult, expensive, and time-consuming processes for clinical trial sponsors (Directive 2001/20/EC). It took 11 years for the EU to acknowledge and attempt to improve the legislation framework. Finally in 2012, the European Commission proposed a revision of the clinical trial legislation in order to strengthen the EU's competitiveness in this important field of red biotechnology. The current Regulation (EU) No 536/2014 of the EUROPEAN PARLIAMENT and of the Council of 16 April 2014 on clinical intervention trials on medicinal products for human use, repealing Directive 2001/20/EC, contributes to the desired harmonization and brings improved efficiency in the regulatory framework for clinical trials, without affecting the high safety standards of patients and the robustness of clinical data (Reg. No 536/2014).

Placing new pharmaceutical products on the EU or EEA (European Economic Area) market requires granting an authorization (Decision No 51/2006/EC) that includes an application submission by the pharmaceutical new product developer to the European Medicines Agency (EMA), which is the European Authority responsible for the scientific evaluation of the safety, efficacy, and quality of the new products. The scientific assessment is conducted by the EMA Committee for Medicinal Products for Human Use (CHMP). The active time for scientific evaluation of the marketing authorization application by the European authorities may take up to 210 days. In the case of Specialized EMA Committees, such as the Committee for Orphan Medicinal Products (COMP) or the Committee for Advanced Therapies (CAT), an additional draft opinion on each specific product's application falling under EMA Committees expertise, is required before the CHMP reaches a final opinion on the granting of the marketing authorization.

Special categories of health care biotechnology are the Orphan medicinal products (OMPs) and the Advanced therapy medicinal products (ATMPs):

- Orphan medicinal products (OMPs) are pharmaceutical products intended for the diagnosis, prevention, or treatment of rare life-threatening or seriously debilitating conditions. The R&D processes which lead to their manufacturing are based on advanced scientific research for sensitive clinical cases and needs additional specific legislation, such as the EU Regulation on OMPs, Regulation (EC) No 141/2000. Through this specific legislative framework, the EU managed to create appropriate environment for the development and authorization of new OMP treatments across the EU, which make a significant difference for rare and very rare diseases (Reg (EC) No 141/2000).
- Advanced therapy medicinal products (ATMPs) are special pharmaceutical products which target gene therapy, cell therapy, or use engineered tissue. Their development requires a long and complex process, which was addressed by the EU with specific regulation on advanced therapies, such as Regulation (EC) 1394/2007. Unfortunately, for ATMPs, the European regulatory framework has not been efficient so far, and there still are significant hurdles in ATMPs European uptake, which must be managed in the future.

In addition to the new pharmacological products authorization process, the EU has created a post-authorization legislative framework, through Regulation (EU) No 1235/2010, to support the pharmacovigilance requirements that improve the data collection after a product approval for marketing (30). For

certain products that require additional monitoring, the EU pharmacovigilance legislation provided a separate EMA Committee - the Pharmacovigilance Risk Assessment Committee (PRAC). PRAC provides feedback to CHMP on pharmacovigilance activities and on risk management systems, according to Directive 2010/84/EU (Directive 2010/84/EU). However, without minimizing the patient safety and the need to provide accurate information to the public, these additional systems must be efficiently managed and supervised, especially by assessing the financial burden and administrative hurdles for the companies.

According to the report on biotechnology in Europe: "The Tax, Finance and Regulatory Framework and Global Policy Comparison", which is a joint report by EY and EuropaBio (Report on Biotechnology in Europe), unless appropriate legislative framework is created that would ease the journey from innovation to manufactured products, the European biotechnology research hub may be at danger of losing the associated innovative products, processes, jobs, and economic growth.

ROMANIAN RESEARCH IN RED BIOTECHNOLOGY - FOLLOWING THE CURRENT TREND OF SCIENTIFIC ADVANCES

Considering the impact of traditions over the wellbeing of a population in a certain geographical area and social acceptance of innovations that are linked to traditional technologies, more and more scientists are considering introducing mature knowledge into innovation development process. Based on the evidence brought by recent publications (Capaldo, 2017), scientist are currently embracing the usefulness of adoption of moderately mature knowledge to sustain the value of moderately mature knowledge-based innovative applications that preserve scientific value and demonstrate sustainable feasibility.

Romanian mature knowledge is now increasingly being adopted from academia following the global trend, although it could not find feasible application in the past and was not able to attract adequate financing along with new scientific results, which need pilot projects to be effectively implemented in new applications. Romanian biotechnology companies are growing collaborations with academia through public-private partnerships and contract research activities by using mature knowledge advancements.

As the academia is always a strategic component of the research and development, the education offer is also an important aspect for the development in biotechnology. The higher education offer in Romania has become increasingly attractive in the recent years, not only for the Romanian students, but also for international students. English and French programs of the most important national universities in the country have been bringing increasing numbers of international students from all over the Europe that mostly attend bachelor's degree programs. The biotechnology educational field is represented by over 20 Romanian universities, providing bachelor's degree, master's degree, PhD and Postdoctoral programs in engineering, biology, agriculture, environment and chemistry (Stanciu, 2010).

Past and present hurdles in Romanian biotechnology development. The biotechnology industry in Romania is still in need for further development. This may be due to the lack of interest of past governments in prioritizing this field, so that very few research programs were granted funding. The main fields of Romanian biotechnology companies are contract-research and manufacturing, veterinarian, medical, environmental, analysis, and diagnostic (Stanciu, 2010). Academic research in Romania is still restricted by difficult access to funding and the limited transfer of knowledge from academia to companies. Moreover, EU funding is not efficiently accessed, despite the fact that absorption of structural funds has been a priority for recent governments, leading to a poor project accessing of the EU funds. Some of the most significant reasons include the weak representation of national companies in European projects, poor information systems and lack of start-up capital for co-financing (Stanciu, 2010).

Another draw-back in biotechnology development in Romania was the so-called "asset-less hyper-competence or competence-less lay-off nearby high-tech assets" (Stanciu, 2010; Vidulescu, 2003). In addition to this, most of the young, passionate researchers are

searching for better paid jobs in a more efficient working environment, in EU, USA, Canada, or UK.

Romanian potential for biotechnology research development. Romanian potential as an attractive destination for biotechnology research development has been advocated by producers of innovative drugs. Companies have grown interest in performing R&D activities in Romania, based on the valuable scientific human resource and on the significant project results despite the lack of financing. Companies such as Amgen Romania is seeking for development of an effective strategy for allocating capital into production facilities for innovative products and are confident that Romania is starting to attract significant investments in the red biotechnology field (Business-review, 2019). This is an important forecast for our country, as Amgen is considered the worldwide research specialized leader in biotechnology and personalized therapies for patients with severe illnesses, focusing on R & D in production of medicines in more than 75 countries worldwide.

Romanian programs for research, development and innovation. Romania has a legislative framework for the Strategy of National Research, Development and Innovation 2014-2020, which was approved by Government Decision no. 929. The main instrument for the implementation of this strategy is the *National Research, Development and Innovation for 2015-2020 (PNCDI III),* approved by Government Decision no. 583/ 22.07.2015. UEFISCDI (Executive Unit for Financing Education Higher Research Development and Innovation) PNCDI III is currently coordinating the following programs (UEFISCDI, CNFIS, 2019):

- Program 1: Developing national R & D system - which aims to help the development of human resource, to increase resource efficiency in public organizations, by developing mechanisms for monitoring and evaluating the quality and relevance of R & D activities; increase the attractiveness of research organizations and their partnerships with scientific international community (UEFISCDI, CNFIS, 2019):
  - Subprogram 1.1. Human Resources;
  - Subprogram 1.3. R & D infrastructure;
  - Subprogram 1.4. Support.
- Program 2: Improving the competitiveness of Romanian economy through research, development and innovation - aims to drive progress on enterprise value chains and partnerships with public universities, by maximizing the added value of innovative goods production (technologies, products, services) based on scientific research (own or outsourced); aims to increase the capacity of companies to absorb the latest technology and to adapt these it to the needs of target markets; creates and enables environment for private sector initiative through entrepreneurship training tools, support for R & D product marketing and establishes partnerships between firms, research organizations and possibly local authorities (UEFISCDI, CNFIS, 2019).
  - Subprogram 2.1. Competitiveness through research, development and innovation.
- Program 3: European and international cooperation - has as main objectives: increasing the international competitiveness of Romanian research in attracting external funding for research; strengthening of national research, development and innovation systems through enhanced international scientific cooperation; supporting Romania's participation in the Framework Program for Research and Innovation EU - Horizon 2020 initiatives Commune Programming (JPI), the European Innovation Partnerships (EIP) on other initiatives, programs, organizations and European and international conventions; providing support for Romania's representation in organizations and pan-European programs and international research; providing Romanian increased visibility in research, development and innovation (UEFISCDI, CNFIS, 2019).
  - Subprogram 3.1. Bilateral/multilateral (excluding the bilateral program with AUF);
  - Subprogram 3.2. 2020;
  - Subprogram 3.5. European and international initiatives and programs;
  - Subprogram 3.6. Support.
- Program 4: Fundamental research and border.

Romanian research in 3D printing technology advances. 3D printing provides the opportunity

to create personalized precision medication to treat patients according to their individual characteristics (genetic background, external factors, history of conditions), as recommended by the FDA: "Providing the right patient treatment, at the right dose, at the right time" (FDA, 2013). 3D printing technologies are close to successful use for the production of pharmaceutical forms with different models and dose levels, in a short time in medical facilities, at affordable prices (Park, 2018; Rahman, 2018). These are considered promising techniques for the precise combination of substances with complex release profiles and providing flexibility in dosing and treatment (Konta, 2017). The 3D printing potential is used in the clinical production and applications of medical implants, organs, and tissues. Biocompatible compounds, cells, and substances are assembled together in complex 3D structures such as tissues and living organs. A model with no faults, similar to the anatomical model, is produced using a high-quality 3D image obtained from the patient to produce the data required for the creation of rapid prototypes of the desired structure (Ozbolat, 2013; Preis, 2017).

Bio-printer 3D works with special ink substances, which must be biocompatible, printable, biodegradable, allowing vascular and nervous regeneration and cell differentiation. In addition, bio-printer inks must be affordable and available in unlimited amounts. Intensive research is thus carried out in the area of biopolymers in Romania, due to their specific properties (Lupuleasa, 2011). Biodegradable polymers have special properties, as they will not induce an inflammatory response, their mechanical properties are designed and pre-established and are cleaved to soluble degradation products, via hydrolytic or enzymatic path, being safely cleared from the body. Currently, scientists believe that 3D printing technologies are capable of overcoming present hurdles in drugs and medical devices manufacturing (Lupuleasa, 2011).

Antibody therapy new product development blooming and Romanian research support.

The recent increasing pace in antibodies - new product development, as reflected by numerous studies (Kaplon, 2018) and revealed by an increasing number of antibody therapeutics grant approval (for phase III and IV of clinical trials), both in the USA and in the EU, has driven a similar trend in the number studies being published in relation to efficiency in various patient categories. The research performed in this red biotechnology application field is also supported by Romanian scientists. For example, in an observational study conducted in Romania concerning the rheumatoid factor (RF) and anti-CPA antibodies, known as negative prognostic factors in the rheumatoid arthritis (RA) treatment, Codreanu et al. (2018) pointed out that the therapeutic significance of the antinuclear antibodies (ANA) in the RA is unclear and aimed the assessment of the possibility of using ANA as a prognostic factor for the therapeutic response to biological RA PR. They included 740 patients with PR and found that patients with positive ANA had significantly higher disease activity score before the biological initiation compared to patients without ANA. They concluded that positivity of the ANA in patients with RA prior to the initiation of biological therapy could be a negative prognostic factor for the effectiveness and persistence of treatment (Codreanu, 2018).

Biological pharmaceutical products in Romania.

Over time, Romanian biotechnology has produced medicines such as insulin, coagulant VIII factor for Haemophilic patients, monoclonal antibodies for targeted therapies as well as for cancer immune therapy, orphan pharmaceutical drugs for rare diseases, vaccines, CAR-T cellular therapies or complex therapies for repairing organs, skin, bone and cartilage lesions.

Despite these advances, there is still a poor market for medical biotechnologies in Romania compared to other countries, hence a lower overall level of information about the implications and the specificity of these therapies. The most common information about bio-based medicines is about comparing original biobased drugs with biosimilar ones, but very little information is found on the specificity of bio-based medicines, patient safety issues and ensuring their traceability.

Due to insufficient analysis of the extent of knowledge of biological medicines or general perceptions of such medicines in Romania,

pharmaceutical companies initiated a research study based on the views of authorities representatives and stakeholders, which has shown that there is a need for more extensive and detailed information (for those who use or regulate them) on the specificity of bio-medicines (Grabowski, 2014; Giezen, 2009).
Therefore, considering the conclusions of consultations held with stakeholders, it would be appropriate to increase the awareness of Romanian decision-makers and stakeholders about biological treatment and their specificity, with a view to increase patients' access to such treatments and safer treatment application (Giezen, 2009).

CONCLUSIONS

Red biotechnology and bio-based medicines have changed and continue to fundamentally change the fate of patients with serious diseases such as cancer, diabetes, haemophilia, rheumatoid arthritis, myocardial infarction, and intestinal inflammatory diseases. At the moment, several hundred bio-based drugs are being developed worldwide, with most of them being cancer medicines. Many medicines are expected to reach the market in the near future, with major challenges for medical practice, for patients, and also for the health system as a whole. The top 5 most productive countries in terms of red biotechnology scientific research were the United States of America, China, Germany, Brazil, and India, while the USA is the world leader in terms of patent application number. On a global perspective, the main hurdle in red biotechnology is lack of financial support for research activities and application of pilot projects.
As European legislation is currently changing rapidly and the needs are increasing accordingly, additional ways to improve communication between scientists, industry, and the society should be found for the desired outcomes of public goods. Financial tools to ensure appropriate climate for research, development, and innovation of biotechnology goods also need to be created. However, the main drawback at the European level remains the legislative framework. Unless appropriate regulations that would ease the journey from innovation to manufactured products are passed, the European biotechnology research hub may be at danger of losing the associated innovative products, processes, jobs, and economic growth.
Even though the biotechnology industry in Romania is still in need for further development due to numerous past and present drawbacks, Romanian potential for biotechnology research development was recognized by important biotech companies as an attractive destination. Currently running large programs for research, development, and innovation, regulated within a national implementation strategy, and with an attractive higher education offer, Romania is contributing to biotech advances, new product development, and has a great potential for production of biological pharmaceutical products, therefore representing a valuable and promising partner for future international collaborations.

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TECHNOLOGIES OF ANIMAL HUSBANDRY

Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

IMPACT OF CULTIVATION METHOD AND AGRICULTURAL LANDSCAPES ON WILD BEES

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Abstract

The aim of this paper is to present economic impact of cultivation method and optimizing resources. The presence of high quality natural and semi-natural habitats on farms and in agricultural landscapes such as: strips of honey plants interspersed in agricultural crops, wooded areas, living hedges and the edges of grassy fields are essential for the survive al of wild bees. The paper scientifically argues why pollination of agricultural crops should be based primarily on wild bee herds that need to be protected. By integrating beekeeping into aquaponics systems, reducing transhumance is transformed from a compensatory measure of production losses into a measure of increasing productivity and stimulating sustainable economic development with positive effects on environmental resources and thus providing the opportunity to conserve biodiversity.

Key words*: agricultural landscapes, bees, aquaponics systems, cultivation method.*

INTRODUCTION

The bees need access to a semi-natural habitat for survival, to ensure nesting sites and also to provide them with pollen feed and nectar from wild flowers. Thus, natural and semi-natural habitats are needed to support bees.

Agricultural and livestock activities are considered the biggest consumers of fresh water. Estimations reveal that 85% of the global fresh water consumption is for agriculture and nearly one-third of the total water footprint of agriculture in the world is used for livestock products (Hoekstra, 2007) and (Mekonnen & Hoekstra, 2012).

Aquaponics is an integrated and intensive fish-crop farming system under constant recirculation of water through interconnected devices.

Aquaculture development as a whole in the country in combination with production technology, favourable socioeconomic condition and culture environment has already proven successful in terms of increasing productivity, improving profitability and maintaining sustainability (Toufique & Belton, 2014).

The landscapes of intensive industrial agriculture do not support the populations of wild bees and also the pollination services they offer. Organic farming has shown that agriculture without synthetic chemical pesticides and biological pest control is viable: Organic farming does not use synthetic chemical pesticides and measures are being used to increase the biological control of pests. These measures include encouraging natural enemies, such as birds, certain cockroaches, spiders and parasitoids, which are means of biocontrol of pests in agricultural crops. Some scientific studies have shown that natural enemies can suppress harmful insects from crops, thus providing a biological means of controlling natural pests. Scientific research has also shown that the diversity and abundance of natural enemies are improved on organic farms.

Honey production and consumption in Europe

The European Union (EU) is the world's second largest producer of honey and plays an important role in the beekeeping market. European honey production covers only 60% of the annual needs of Europeans. Honey consumption at European level is about 20-25% of world consumption, being 0.70 kilograms per person per year. So the EU is one of the largest importers in the field, with annual honey imports ranging between 120 000 and 150 000 tons. The main suppliers are China,

with 63 900 tons (43% of total EU imports), Argentina with 22 300 tons, Mexico with 21 200 tons, and Ukraine with 8 900 tons of honey. Also, the low price of bee products in China leads to lower exports from EU member states. Support for European apiculture in recent years, the EU has implemented support programs and policies for beekeepers. These measures have taken into account the problems faced by European beekeepers, namely the massive loss of bee colonies, honey production costs and fierce competition in the market. The progress of beekeeping will contribute to increasing the competitiveness of this sector and the economic development of rural areas, and by pollination, bee colonies will continue to act as providers of important environmental services, ensuring the sustainable development of these areas.

Scientific research shows that a diversity of untamed bee species is preponderating for guaranteeing property crop production.

Thus, we tend to cannot believe alone on one species - managed honey bees - for impregnation.

A diversity of untamed bee species is additionally essential to confirm food is delivered to our tables each day.

Recent scientific studies have shown that chemical intensive industrial agriculture is involved within the decline of bees and therefore the impregnation services they supply to our crops and wild flowers.

Ever increasing applications of fertilisers, herbicides and insecticides and their synergistic negative impacts on bee health and loss of natural and semi-natural habitat on field, farm and landscape levels are major drivers of bee declines.

Further, the fashionable industrial farming model conjointly causes issues of growing resistance of pests and weeds, diminished soil fertility and water retention, contamination of ground waters, high energy input and CO_2 emissions, as well as reduced resilience and increased vulnerability to climate change.

The analysis of these factors allows the measurement of:

• the decline of bee colonies;

• identification of causes and solutions;

• lack of access to the results of applied research;

• insufficient understanding of the economic opportunities offered by the diversification of the production of the integration of more horizontal and vertical activities and of the marketing and other products, other than honey (Figure 1).

As a planning tool and guide in the development of a business, the business plan ensures the knowledge of the current state and prospects of the company in competition with other partners in the case of making an investment of common interest or the realization of an association.

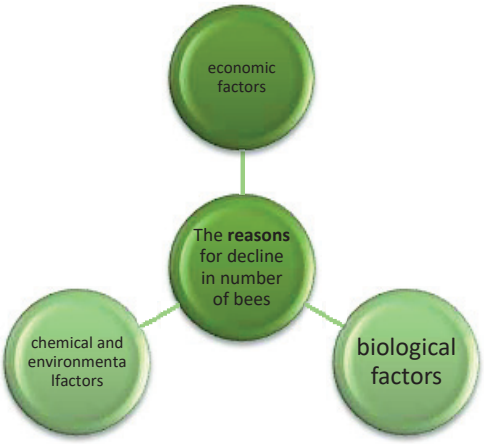

Figure 1. The reasons for decline in number of bees in the near past

MATERIALS AND METHODS

The support granted to support European beekeeping in recent years by the EU's common agricultural policy (CAP) has been represented by a series of policies that have implemented programs and support measures for beekeepers. These measures of the common agricultural policy, considered, a series of problems with which the European beekeepers frequently face, namely, the massive loss registered within the herds of the bee colonies, the increase of the production costs for honey bees and the fierce competition existing on market. The main objectives of all these programs and measures have been permanently improved through actions in the level of production technologies and through actions in the field of marketing of bee products. We can

mention the following: the support programs that include technical assistance (training courses), the active measures for combating varicose veins and at the same time the peaceful measures represented by the rationalization of the transhumance and the support granted for analyzing the quality of honey and developing research in the field of intensive beekeeping. In Europe, we find that the number of bee hives has registered a strong long-term decline, while the quantity of honey production has remained quite stable. The number of bee hives decreased by 25% between 1965 and 2016. This decline in the number of bees began in particular around 1985 and has stabilized to a great extent since 1995. The reasons for this decline mentioned both in the literature and in official documents, as well as in interviews and surveys, are:

• economic factors (reduced profitability due to import pressure and increased purchasing power of large middlemen, such as wholesalers and retailers);
• biological factors (pests and diseases);
• chemical factors (insecticides and pesticides);
•environmental factors (anthropogenic degradation).

This is a synthesis paper, which aims to show the impact of cultivation method and agricultural landscapes on wild bees, for sustainable management of vegetal and bees resources, in fact for life on the Earth. In this regard, a series of recent scientific studies were used, in order to emphasize all the main characteristics of, bees and environment stressors. This study employed qualitative technique for examining connections and interactions between varies mechanisms, human and agri-companies activities regarding bees.

Mathematical models within the study of environmental phenomena carry on with the newest ends up in the mathematical domain that might offer solutions for dominant, analysing, predicting and study of risk phenomena.

Water quality models usually consist of a set of mathematical expressions relating one or more water quality parameters.

In any set of environmental measuring, the subjects of accuracy and precision of the measurements are always beneath the surface. Most environmental discharge permits embody ordinarily distributed statistics for environmental events.

This is incorrect and rarely accomplished. The Mathematical model of the evolution water quality parameters and the accepted mathematical structures can help establish a more comprehensive map of risk factors. According to the ISO 31000 Risk Management Standard 2009, risk is simply "the effect of uncertainty on the objectives". Based on this definition, the phrase "everyone manages risk" is therefore true. If we accept that all individuals and organizations have goals, that these goals are necessarily set in the future and that the future is uncertain, then each and every organization manages the risk. The risk management system provides tools to build a structured vision of the future and to address the issue of related uncertainty. Implementing risk management in an organization or regulatory body gives management the opportunity to make rational decisions based on available information, no matter how full it is. In order to prove the feasibility of implementing a risk management system, we will return to the fundamental principle of project management, which characterizes the interdependence of the following parameters: the budget, the quality of the finished product and the implementation time. Risk management tools help to make a rational choice among a number of alternatives

In other words, the level of achievement of the desired regulatory objective will depend on the cost of the preventive measures and the abandonment of the expected benefits from one or more areas of economic activity. To identify the risk, the project team should review the scope of the program, cost estimates, program (including critical path evaluation), technical maturity, key performance parameters, performance challenges, stakeholders' expectations of the current plan, external dependencies, and internal challenges, integration, interoperability, sustainability, supply chain vulnerabilities, threat management, cost deviations, test event expectations, safety, security, and more. The analysis of the factors influencing entrepreneurship risk in the beekeeping sector was made using the research method as

research method, and the research tool chosen is the questionnaire. The reason for choosing this method is the desire to get the most relevant information about the factors of influence of entrepreneurship risk in the beekeeping sector in the North-West Region and the extent to which beekeepers have succeeded or intend to access European funds. The questionnaire is designed to get as much data as possible on the factors that influence a beekeeper's decision to start a beekeeping business, the level of knowledge and education, the determination of the degree of access to European funds, the collaboration with companies in beekeeping sector, etc. The purpose of applying the questionnaire is to find out the link between the beekeepers' socio-personal characteristics and their strategies and the intention to start a business in the apiculture sector. After the investigation, a centralization of the questionnaires was carried out, followed by data interpretation and analysis.

RESULTS AND DISCUSSIONS

The agricultural policy measures related to technical assistance in beekeeping have decisively contributed to the increase of productivity and quality. The technical assistance was mainly provided through professional training measures, allowing the dissemination of technical information with novelty character among all beekeepers and facilitating the purchase of the latest equipment that offers efficient solutions for honey production and for obtaining other bee products.

Analysing the impact on production determined by the measures that support the fight against the *Varroa* parasite, a virus that is a major threat and which requires high costs, the European Commission considers that the effect of these measures has been very positive. Although positive results have been obtained in some EU Member States, the adoption of measures has been limited. These deficiencies found in the fight against varicose veins were due to the fact that the procedures required to obtain the assistance needed to combat varicose veins were considered difficult by the beekeepers. One of the most effective measures has been shown to support the rationalization of transhumant. This measure has been highly appreciated in the case studies conducted in Greece and Spain. Therefore, we consider that the extension of this measure can be applied in intensive beekeeping systems integrated within an aquaponic system. Thus these super intensive production systems offer the opportunity to increase productivity and reduce the level of transhumance, while also providing support in controlling the spread of the *Varroa* mite. The economic efficiency of this measure is obtained through the integration of several production branches both horizontally and vertically within the same production capacity, without introducing additional production factors. This measure of rationalization of transhumance has been rarely used elsewhere in the EU, because the measure is more suitable for professional beekeepers with a large number of hives. By developing intensive and integrated beekeeping systems, it is thus possible to apply this measure of streamlining of transhumance and for beekeepers with small bee numbers who can achieve a considerable increase in beekeeping income against the background of its location within integrated aquaponics systems.

When the bee hive repopulation measure was applied, those beekeepers who received general support recorded a positive effect on production. The repopulation of agricultural land with wild bees specialized in pollination of crops would also generate positive effects for all other branches of agricultural production. The associations and the individual beekeepers who were consulted in the evaluation of the effects of the policy measures applied within the beekeeping sector at the European level, unanimously expressed their firm conviction regarding the potential importance of the research measure applied to honey production.

An aquaponics system requires frequent attention (Figures 2 and 3). Even on a small scale, aquaponics systems are complex due to their multiple components and requirements. Disease prevention, water level control, and preventing rodents and other problems require

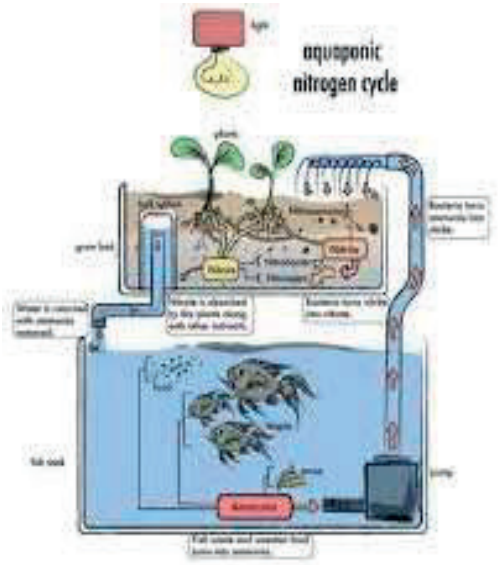

Figure 2. Work principle of aquaponics
Source: Researchgate
Aquaponics Plans (2009). Creating a Sustainable Food Supply Through Aquaponics. Available online at:http://aquaponicsplan.com/creating-a-sustainable-food-supply-through-aquaponics

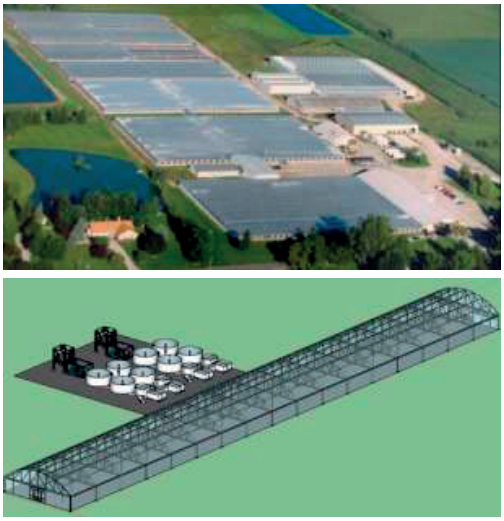

Figure 3. Commercial aquaponics system
Source: Philippines, Bees NEST Consulting Group INC

Harvesting and packing vegetables are also quite labor intensive (Tokunaga et al., 2015) estimated that labor costs were 46% of total operating costs and 40% of total annual costs. This is quite high compared to other forms of aquaculture and prospective aquaponics managers must be certain to have an adequate supply of labor to cover these needs.

Pond water quality is largely defined by temperature, transparency, turbidity, water color, carbon dioxide, pH, alkalinity, hardness, unionized ammonia, nitrite, nitrate, primary productivity, biological oxygen demand and plankton population (Bhatnagar and Devi, 2013).

The accepted level of ammonia should be under the range of 0.05 to 0.10 mg/l (Shoko et al., 2014) and above range it is toxic to the cultured fish.

According to Mizanur et al. (2004), intensive aquaculture ponds sediments has various fertilizing components such as nitrogen, phosphorous, sulphur etc. which are very useful for growth and production of aquaponic plants. Moreover, water spinach is an efficient plant having clustered roots that can absorb nutrients from the water very efficiently according with Kibria & Haque (2012).

It is considered a promising technology, which is highly productive under correct set up and proper management Lal (2013). First, fish feed is eaten by fish and converted into ammonia (NH_3). Some ammonia ionizes in water to ammonium (NH_4^+). Then, bacteria (*Nitrosoma*) convert ammonia into nitrite (NO_2^-) and consequently bacteria (Nitrobacter) oxidize nitrite into nitrate (NO_3^-) (Tyson et al., 2011).

The smaller scale systems had annual net returns that ranged from $4,222 to $30,761. Rates of return on the investment (IRR and MIRR) ranged from 0 percent to 27 percent. Of the studies reported (Tokunaga et al., 2015) is the only one based entirely on a detailed cost analysis of commercial operations. Their analysis showed an MIRR of 7.36 percent, as compared to a cost of capital of 6 percent, demonstrating economic feasibility. The (Tokunaga et al., 2015) profits are lower than those of a number of other studies, but it is not uncommon for analyses with data from commercial farms to show lower levels of profitability than analyses based on hypothetical or experimental data.

Savidov also discussed food safety concerns expressed by consumers over aquaponics produce (Savidov, 2004).

We proudly found that Romanian beekeeping holds the first place in the EU from the

perspective of total honey production. However, the level of production intensification is below the European average, twice lower than the most competitive production systems in Europe, which are found in Finland, Germany and the United Kingdom. This extraordinary economic potential that beekeeping has in Romania contrasts with the lack of competitiveness that manifests itself in the economy as a whole. Through this doctoral thesis I propose an integrated production model that offers advantages related to the intensification of beekeeping production and the increase of competitiveness within the agri-food system. Intensive beekeeping integrated with aquaponic systems is a sustainable and extremely profitable economic activity. With small investments for the acquisition of aquaponic microsystems, significant increases in honey and vegetable production are obtained, thus eliminating the impact of climate change. The investments for the acquisition of an integrated aquaponic microsystem are 250 euros. They shall be recovered from the first year provided that the costs of transhumance are eliminated. Reducing transhumance will lead farmers to adopt biodiversity conservation practices to increase populations of wild pollinators.

CONCLUSIONS

Through this measure of integration of beekeeping in aquaponics systems the measure of transhumance reduction is transformed from a compensatory measure of production losses, to a measure of productivity growth and of stimulating sustainable economic development with positive effects on environmental resources and which thus offers the possibility of biodiversity conservation. The pollination of agricultural crops should be based primarily on the herds of wild bees that should be protected and reintroduced within the agro-ecosystems through specific measures.

For this vision to take effect, it is necessary to start with educational awareness, with the financial support of this vision and with programs to build an adequate human and material infrastructure.

stimulating sustainable economic development with positive effects on environmental resources and which thus offers the possibility of biodiversity conservation. The pollination of agricultural crops should be based primarily on the herds of wild bees that should be protected and reintroduced within the agro-ecosystems through specific measures.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

AGRICULTURAL ADVISORS TRAINING NEEDS - THE CASE OF ALBANIA

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Abstract

This is an exploratory survey, aiming at assessing agricultural advisors training needs and their priorities. Data were collected through a questionnaire mailed to the 66 advisors and the method of data analysis used was the descriptive statistics. All advisors reported they needed more trainings on extension methods & activities, and agricultural practices before they could disseminate the innovations. 67-86% of the advisors stated as important and very important the trainings on extension methods and activities, while 84-90% of them ranked the agricultural practices as important and very important. Less experienced advisors are more interested in the agricultural practices compare with more experienced ones. The majority of respondents (59.1%) indicated that they prefer to receive in- service training during spring. Most of the advisors (63.6%) indicated that the main reason for training is to get acquainted with new technologies to disseminate them to the farmers. The advisors would prefer to receive in-service training on new technologies from the Agricultural Technology transfer Centres (63.6%) and from Agricultural University on plant protection (42.9%).

Key words: *agricultural advisors, Albania, training needs, survey.*

INTRODUCTION

Agriculture is still a significant sector of the economy of Albania, which contributes to about 18% of the country's GDP (World Bank, 2020) and 37.4% of total employment, during 2018 (INSTAT, 2019). This sector is also important in terms of alleviating poverty (where the majority of the lower income population is located in rural areas), improving the standard of living, and one of the main sources of income for rural households (Bicoku & Subashi, 2020).

Albanian agriculture is facing several challenges starting from the small farm size (1.26 ha), and fragmentation of land (about 4 plots per farm)[1]; lack or weakness of farmers' organizations; limited access to agricultural credit; limited access to markets and low standards of products; inefficient farm management practices; and all these weaknesses lead to low level of competitiveness of agriculture (Gjeçi et al., 2018). Several of these weaknesses, such as the low technology level of farmers, or the public and private advisory services not at the level required by farmers, have continued over the last 20 years.

Frashëri (1936) cited by Bicoku and Subashi (2020) pointed out that the beginning of the advisory service in Albania dates back to 1936[2].

During socialist period, with the establishment of agricultural cooperatives and agricultural state farms, this service was covered by the agronomist and the livestock experts of those entities, whose were in charge to train the workers for the daily work and new technologies (Bicoku & Subashi, 2020).

[1]The land reform implemented after August 1991, in which the state agricultural land was equally distributed to the rural population, resulted in small and fragmented farms that hamper the growth and competitiveness of agriculture.

[2]Bicoku, Y. and Subashi, A. (2020): Preliminary Data on Information Source for the Farmers- The Case of Albania. Paper presented in the International Conference "Agriculture for Life- Life for Agriculture", Bucharest, Romania, June 4-6, 2020.

The Albanian advisory service in operation started in 1992, and for a one-decade was supported with technical assistance by donors financed projects. The agriculture advisors were trained with the concepts and principles of extension service and communication. Since March 2018, the extension service organization is organized in four regions (with centres in Tirana, Korça, Lushnja and Shkoder) called the Regional Agencies of Agricultural Extension (RAAE).

Despite improvements in some private and public services, most services are not in support of the time farmers' demands. Skreli et al. (2014), emphasis that the impact of government/public extension service on farm performance is limited, and also the coverage of public extension services is limited, especially towards the contact farms[3], most of which are categorized as medium farms while the private advisory services are the main source of advice for largest farms.

One of the key factors in the advisory process is the education and through it the farmers receive technical knowledge and information, which helps them make decisions about the future of the farm. However, before starting any advisory program it is important to evaluate the knowledge of extension agents toward the innovations that need to be disseminated (Al-Shayaa et al., 2012).

According to Oakley and Garforth (1985), extension is the process through which knowledge is communicated, in various ways, to farmers, through extension agents. But in order to do this job extension agent need to be trained. One aspect of this training is to provide to the extensionists the technical knowledge needed for their job. This is usually done during the professional training of the extensionists; however, this is not enough as the extensionists in addition to technical knowledge must know how to communicate this knowledge and how to use this knowledge for the benefit of farmers.

Erbaugh et al. (2007), Khan et al. (2011), and Man et al. (2016) are emphasizing that training is one of the most important tools to bring out the best of employees, because it fosters their morale, enthusiasm, and interest in their job. As many agricultural extension organizations worldwide face challenges of professional competence among their employees, it is needed the systematic training with the aim to develop knowledge, skills and behaviour, which are the basic requirements for performing a certain job or task. Whereas, the lack of training, after being employed, by the agricultural extension organization, affects negatively the success of the work of the extension workers especially for the planning of the annual work program.

Several authors (Bradfield, 1966; Maunder, 1972; Easter, 1985; Randavay & Vaughn, 1991; Najjingo-Kasujja & McCaslin, 1991) pointed out that extension agents need technical and professional skills and competencies to design, implement, and evaluate educational programs for farmers. The lack of a proper balance between technical and professional competencies in staff has been identified as a problem in the extension services organizations of developing countries. One of the weaknesses in the past, in the training of extension staff, has been the inability to focus on the development of professional competencies. Extension agents, in developing countries, should have professional competence in the areas of program administration, planning and execution, evaluation, communication, teaching and extension methods, and understanding of human behaviour.

It is also argued that co-production knowledge, for example, between farmers and advisers, is a new form of knowledge, combining scientific evidence and training, technical information, experience-based knowledge, information on household goals and interests, the unspoken knowledge of farmers, etc. This shows that agricultural advisory services are characterized by diversity and complexity. It is therefore argued that it is necessary to combine extension methods to increase knowledge transfer and improve learning in agriculture (Labarthe & Laurent, 2013).

While Karbasioun (2007) is emphasizing that raising awareness of good practices and motivating farmers is an important part of extension agents and extension services organizations. Extension can make a significant contribution to the sustainability of agricultural production and rural development, especially

[3]Farmers with whom the advisers have regular contacts.

when the spread of new agricultural technologies to farmers is accompanied by technology education; there is a critical need for a large number of well-trained extensionists in many developing countries (Omoregbee & Ajayi, 2009).

Allo (1983), as well as Yondeowei & Kwarteng (2006), have defined the need for training as the difference between the required level of individual competence and the current level. They added that one of the main factors limiting the effective development of training programs for agricultural advisors and agricultural experts in developing countries is the lack of information on their training needs. Agricultural advisors in developing countries, do much more work than just visiting the farm and telling the farmer about a new technology.

MATERIALS AND METHODS

The purpose of the survey was to identify training needs of agricultural advisors on technical aspects as well on extension methods and communication.

The preparation of the survey has been made possible by the use of primary, secondary sources and literature data related to extension service in the field of agriculture.

The survey was conducted with 66 extensionists (all of who have a computer, about 90% of the staff)) whom are staff of RAAE of Tirana and Korça.

For the purpose of this survey, a questionnaire is designed for interviewing extension agents and collecting the data needed. The questionnaire consists of a series of questions. There are questions about the extensionists personal background, such as age, gender, year of graduation, working experience, education background. Other variables in the dataset relate to training, including questions about most appropriate time of year for you to conduct the trainings; the reasons why the trainings will serve to the extensionists in the future; and how they prepare the work plan and who approves and changes it.

A structured questionnaire was developed to assess extensionists' previous trainings on extension methods and communication, as well as technical issues; and the most important part of questionnaire were the questions about training needs of the extensionists for the future. The questionnaire was subject to review by a panel of two experts, which was conducted via Google Meet[4]. In addition, the questionnaire was pre-tested with a pilot group of the extensionists, which was done also via Google Meet; which in case of inconsistent questions, it was modified accordingly.

The survey was administered in early May 2020, and the questionnaires were filled electronically by the extensionists, as Covid-19 protocols didn't allow the direct interviews. The extensionists were clarified about the purpose of the interview and the survey, as well that the data would be confidential.

The questionnaire contained open-ended and closed-ended questions. Open-ended questions allow for a greater variety of responses from participants, but are difficult to analyse statistically because data has to be coded or reduced in some way. While, closed-ended questions are easy to analyse statistically, but they seriously limit the answers that participants can provide (Jackson, 2009). Also, a Likert-type scale (1932) is used because it is very easy to analyse statistically and it is commonly used in agricultural research (Clason & Dormody, 1994).

The data obtained were entered in Microsoft Excel and transferred into SPSS. The analysis is based on descriptive statistics, namely frequencies.

RESULTS AND DISCUSSIONS

1. General data on the interviewed agricultural advisors

The purpose of this study was to identify the training needs of the experts of the public advisory service of the RAAE of Tirana and Korça.

As can be seen from the data in Table 1, 62% of the interviewees interviewed are male and 38% female (63% and 37% of RAAE Tirana and 61.5% and 38.5% of RAAE Korça).

The extensionists have a long working experience in agriculture (23 years as average; 26.5 years of RAAE Tirana and 20.7 of RAAE Korça), but only 11.3 years in extension (11.7 years in Tirana and 11.0 in Korça); difference

[4]The Covid-19 protocols didn't allow the direct interviews and meetings.

which is explained by the frequent movements made in the direction of the Ministry of Agriculture and the Governments.
In terms of education, 62.1% of extensionists have a degree in agronomy, 22.7% in animal science, 6% in plant protection and by 3% in fruit growing, in agrarian economics, and in agro-environment (Figure 1).

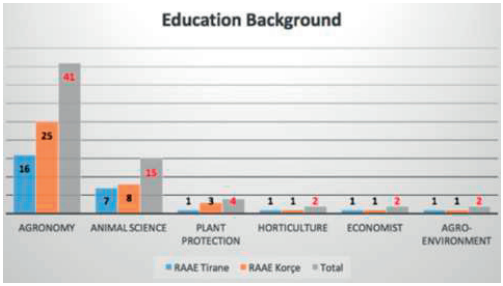

Figure 1. Extensionists education background

All extensionists respond that they prepare extension work plans, most of them monthly, and very few weekly and daily plans (Table 2).
For the preparation of extension work plans the main opinion is of contact farmers, but what impresses is that more opinion is taken from large farms compared to small farms (89.4 versus 40.9%) at a time when in the vision of extension organizations is the support of small and medium farmers (VKM, 2014). Only 8% of extensionists say the extension program is the same as last year, while others say there are changes. In addition, 50% of them report that the supervisor makes changes in the annual plan which is in the level of 14.7% (16.5% in Tirana and 11.7% in Korça). The main changes (reported by 88% of extensionists) are to adapt the objectives to the requirements of the farmers.

2. Topics in which extensionists have been trained in the last three years (2017-2019)

Most extensionists (90.9%) answer that they have participated in trainings during the last three years. For the period 2017-2019, in terms of training conducted on topics related to extension (28 topics related to communication and methods used in extension service), only 0-50% of extensionists have been trained.
What should be emphasized is that for some topics extensionists were not very interested (57-70%) despite the fact that in the last three years very few of them have attended training on topics such as: *"Knowledge of why people come together in groups or associations"; "Knowledge on types of communication"; "Knowledge of data retention"; "Knowledge to present results of surveys"; "Knowledge to conduct surveys"; "Knowledge to identify local leadership"; "Ability to organize study-tours"*.
The extensionists were not trained in data retention, to conduct surveys and to present the results, so it's not clear how they can make accurate annual planning of their activities.
While, in terms of trainings conducted on technical issues related to agricultural production (9 topics) the results are completely different, where the interest of extensions has been 10% higher than in extensions related topics.
As with the technical topics of agricultural production, even in those related to animal production, the participation of extensionists in training was from 26 to 58 percent.
For technical topics related to livestock, fewer extensionists were trained compared to the topics for agricultural production and extension. Perhaps this is explained by the fact that 71.2% of extensionists have agronomy education.
For technical topics of agriculture and livestock 81.8% of extensionists consider them important and very important, compared to 76% who consider extension and communication topics.

3. Topics in which extensionists are interested in future training.

According to extensionists, knowledge related to communication techniques (*"Knowledge of communication types", "Knowledge to motivate farmers", "Ability to organize focus group discussion", "Ability to determine customer needs"*) qualifies as very important and significant to the extent of 67-76%. While knowledge related to extension methods and farm management (*"Knowledge to solve farmers' problems", "Ability to organize demonstrations", "Preparation of Extension Activities Program", "Knowledge on farm management"*) are considered very important and significant to the extent of 80-86% (Figure 2).
Al-Rimawi et al. (2017) and Raad et al. (1994) also report "*Extension Activity Programming*"

as the most important. Whereas, in the study of Al-Zahrani (2017) the extensionists have considered as the most important the "*Ability to motivate farmers*" and "*Knowledge for problem solving*". Omoregbee and Ajayi (2009) reported "*Demonstration planning*" as the most important topic for extensionists.

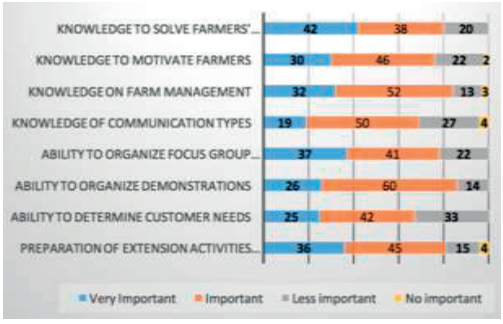

Figure 2. Training topics required for communication and extension methods

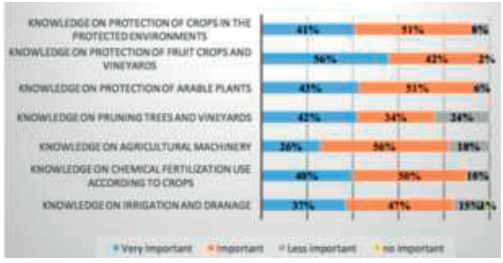

Figure 3. Training topics required on agriculture

According to extensionists, knowledge related to technical topics of agriculture (*"Knowledge on irrigation and drainage", "Knowledge on chemical fertilization use according to crops", "Knowledge on agricultural machinery", "Knowledge on pruning trees and vineyards"*) is considered very important and significant to the extent of 84-90%. While the knowledge related to plant protection (*"Knowledge on protection of arable plants", "Knowledge on protection of fruit crops and vineyards", "Knowledge on protection of crops in the protected environments"*) qualify them as very important and important at the rate of 92-98% (Figure 3). The same result is reported by Chizari et al. (1999); Haleem (2018) and Man et al. (2016), where the interest of extensionists in the first place is for the integrated management of pests, as well as the vegetables' diseases control in protected environments. While Ommani & Chizari (2009) report problems related to irrigation and water use efficiency as the main problems. In the study of Man et al. (2016) an important topic for the training of extensionists is the use of fertilizer in agricultural crops.

As can be seen from Figure 4, the extensionists consider as less important the topics related to livestock in the amount of 11-37%, where the preparation of silage and hay is of less interest ("Knowledge on hay preparation", "Knowledge on silage preparation", "Knowledge on animal feeding and nutrition", and "Knowledge on major animal diseases such as zoonoses, mastitis, parasitic diseases").

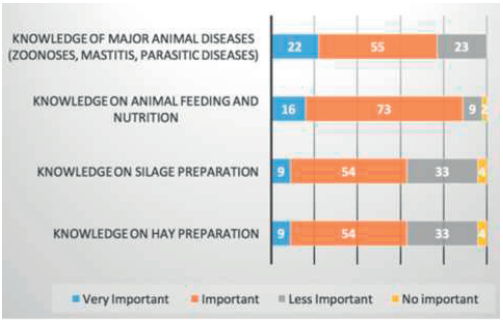

Figure 4. Training topics required for livestock

From all the topics reviewed it turns out that for plant protection are the greatest demands for training and this is explained by the multitude of plant diseases and fruit growing, as well as by the preparations for combating these diseases, which are constantly changing. Even in the study of Man et al. (2016) the most important topic for the training of extensionists, in livestock issues, are the skills for animal diseases followed by and after that animal feeding and nutrition.

3.1 Topics in which extensionists are interested in future training (according to the education background)

As we can see from Figure 5 there are differences between agronomists and zootechnicians in terms of the training they need on topics related to agronomic techniques. These topics are less important for agronomists (7-38%) compared to zootechnicians (7-18%).

Less important topics for agronomists are those related to irrigation-drainage and pruning of fruit trees / vineyards, while for zootechnicians irrigation-drainage and fertilization by crops.
From Figure 6 we notice that there are differences between agronomists and zootechnicians in terms of the training they need on zootechnical issues. All zootechnicians consider them very important and important while agronomists 17-30% say that they are less important. This is explained by the fact that agronomists are mainly engaged in advising farms with crop production and fruit growing.

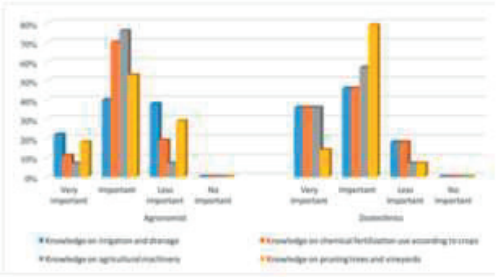

Figure 5. Training topics on agriculture - agronomist vs zootechnicians

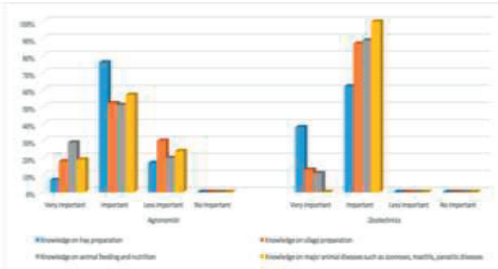

Figure 6. Training topics on livestock - agronomist vs zootechnicians

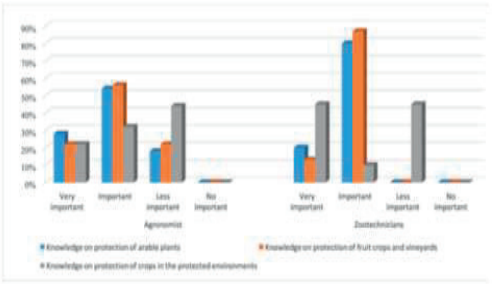

Figure 7. Training topics on plant protection - agronomist vs zootechnicians

The data of Figure 7 shows that zootechnicians are very interested in topics related to the protection of field plants (100% of them compared to 82% of agronomists), as well as the protection of fruit growing and vineyards (100% of them compared to 78% of agronomists), while regarding the topic of plant protection in protected areas in the categories of specialists are less interested, to the extent of 54-55%. The greatest interest of zootechnicians in agronomic topics is explained by the lower knowledge they have about these particular topics.

3.2 Topics in which extensionists are interested in future training (according to working experience in extension)

There are differences between the two groups of extensionists according to the working experience in extension in terms of training they need on zootechnical issues (figure 8). Less experienced extensionists consider less important topics for hay and silage preparation (39 and 45%) compared to more experienced extensionists (33-26%). As can be understood these specialists think that they deal mainly with advising farms with crop production and fruit growing.
Chizari et al. (2006) in their study report the same thing, that the more years in extension the extensionists think that their training needs are not very important.

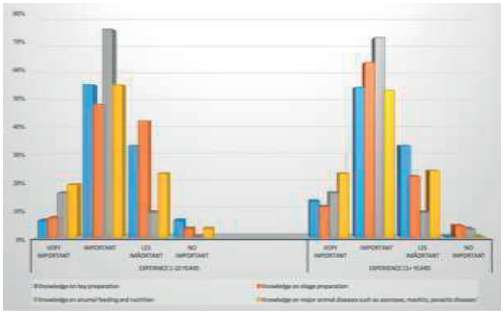

Figure 8. Training topics required for livestock technical problems (extension experience)

According to the experience in extension, in terms of training they need on topics related to agronomic techniques, there are differences between the two groups of extensionists (Figure 9). Thus, in terms of “Knowledge for irrigation and drainage” and “Fertilization according to agricultural crops” are less

important for the group with less experience (20% and 16%) compared to the group with more experience (10% and 4%). As for the "Knowledge of pruning trees and vineyards" 87% of the less experienced extensionists consider it very important compared to 75% of the more experienced extension group, and this explains that the more experienced extension group has performed trainings on this topic.

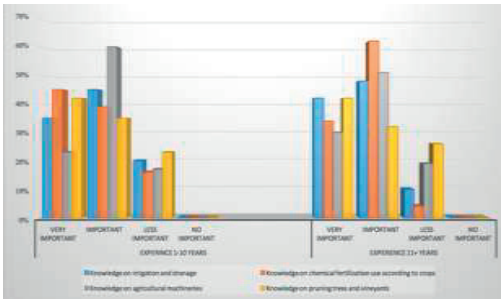

Figure 9. Training topics required for agronomic techniques (extension experience)

All extensionists with less experience (100%) are interested in "Knowledge for the protection of fruit growing and vineyards", this explains that 62% of these extensionists are from AREB-Korça. As for "Knowledge about the protection of field plants and protected areas" there is not much difference between these groups (Figure 10).

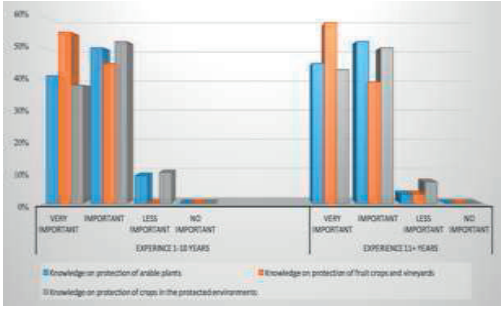

Figure 10. Training topics required on plant protection (extension experience)

So, from the data of the figures above, it appears that for the topics relating to technical problems of agriculture and livestock 80.5% of the group of inexperienced extensionists considers them important and very important, while only 76% of the group of experienced extensionists qualifies them as important.

But what is not expected is that the group of less experienced extensionists considers the topics related to communication and extension important and very important to the extent of 73.4% against 78.1% of the group with more experience (Figure 11).

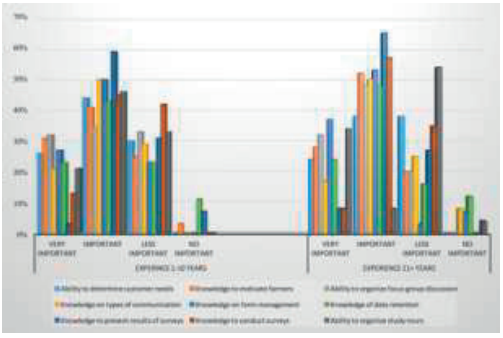

Figure 11. Training topics on extension topics (extension experience)

4 The best period of acquiring new knowledge for extensionists

In terms of the most suitable time for training, most extensionists selected spring, and then autumn, while for winter (7.6%) and summer (9.1%) only 1/6 of them have this preference (Table 3).

Interestingly, the extensionists responses of both RAAE are almost the same. While authors like Chizari et al. (1999), report winter as the most suitable period for training.

5. Three reasons for the purpose of training

Another question addressed to the extensionists was what were the three reasons for the purpose of the training (Table 4). The extensionists of both RAAE have the same answers as it is "To get acquainted with new technologies and to pass them on to the farmer" (41.2% of the total) and "Training on technical issues and updating knowledge" (36.3% of the total), but also have differences for the third reason where the Tirana extensionists mentioned "To be able to convince farmers of economic growth" (7.8%), while in Korça "To communicate as freely as possible with farmers and contact groups" (14.7%).

Table 1. Main sample socio-demographic

RAAE	Extensionists											
	Number of interviews	Gender		Age (years)	Experience (years)		Education					
		M	F		Total	In extension	Agronomy	Zootechnist	Plant protection	Horticulture	Economist	Agro-environment
Tiranë	27	17	10	53.6	26.5	11.7	16	7	1	1	1	1
Korçë	39	24	15	51.7	20.7	11.0	25	8	3	1	1	1
Total	**66**	**41**	**25**	**52.5**	**23.1**	**11.3**	**41**	**15**	**4**	**2**	**2**	**2**

Table 2. Preparation of extension work plans

RAAE	Are extension work plans prepared?					Whose opinion is taken for the preparation of plans?				
	Total	Annually	Monthly	Weekly	Daily	RAAE	Colleagues	Farmers:		
								Contact	Large	Small
Tiranë	27	20	27	2	5	22	13	26	23	10
Korçë	39	39	38	5	6	33	9	39	36	17
Total	66	59	65	7	11	55	22	65	59	27
%	100	89.4	98.5	10.6	16.7	83.3	33.3	98.5	89.4	40.9

Table 3. The most appropriate time to acquire new knowledge for extensionists

RAAE	Trainings most appropriate time							
	Winter		Spring		Summer		Autumn	
	Person	Percent	Person	Percent	Person	Percent	Person	Percent
Tiranë	2	7.4	17	63.0	2	7.4	6	22.2
Korcë	3	7.7	22	56.4	4	10.3	10	25.6
Total/average	5	7.6	39	59.1	6	9.1	16	24.2

Table 4. Three reasons for the purpose of training

RAAE	**Main reasons for the training purpose**		
	To get acquainted with new technologies and to pass them on to the farmer.	Training on technical issues and updating knowledge	To be able to convince farmers of economic growth
Tiranë	**54.1%**	**24.3%**	**21.6**
	To get acquainted with new technologies and to pass them on to the farmer.	Training on technical issues and updating knowledge	To communicate as freely as possible with farmers and contact groups
Korçë	**33.8%**	**43.1%**	**23.1%**

CONCLUSIONS

Our survey shows that we have a gender disproportion in the ranks of extensionists, where 62% are male and 38% female, at a time when female extensionists had to be at least 50%, since most farming work is performed by women.

Extensionists have a long work experience of 23 years but only 11.3 years in extension, which tells us about frequent movements of extension staff, which negatively affects not only the work of the extensionists but also farmers.

Disproportion is also observed in employment by profession where 62.1% of extensionists are agronomists, and only 22.7% zootechnical, when livestock production provides about 52% of total agricultural production

For the preparation of extension activities plans the main opinion is of the contact farmers, but what negatively impresses us is that more opinion is taken from the large farms compared to small farms (89.4 versus 40.9%).

Most of the respondents (90.9%) answered that they have participated in trainings during the last three years, but for the topics related to communication and the methods used in the advisory service very few of them have been trained.

What attracts attention is that for some topics the extensionists are not very interested despite

the fact that in the last three years very few of them have attended trainings for them (Knowledge of why people join groups or associations; Knowledge of forms of communication; Knowledge for data retention; Knowledge to present results; Knowledge to conduct observations; Knowledge to identify local leadership; Ability to organize study tours). This shows that extensionists are not trained in keeping records, conducting observations or presenting results and how they can make accurate annual planning of their activities.

For technical topics the interest of extensionists to be trained is 10% higher than in topics related to extension. The interest of extensionists to be trained in livestock production issues is lower compared to the topics of agricultural production and extension. Perhaps this explains because about 75% of extensionists are agronomists.

For future training, extensionists consider as very important and relevant topics related to extension methods and farm management and then knowledge related to communication techniques. According to extensionists, topics related to knowledge of plant protection are more priority than technical topics of agriculture (such as irrigation and drainage, use of fertilizer by crops, agricultural machinery, pruning of trees and vineyards).

From the interviews it appears that, the extensionists consider as less important the topics related to livestock, where with little interest are the preparation of silage and hay.

So, from all the topics reviewed it turns out that the highest demand is for plant protection training and this is explained by the multitude of plant diseases and fruit growing, as well as by the preparations for combating these diseases, which are constantly changing.

We notice that there are differences between agronomists and zootechnicians in terms of the training they need on zootechnical issues. All zootechnicians consider them very important and important, while 17-30% of agronomists say that they are less important. This is explained by the fact that agronomists deal mainly with advising farms with crop production and fruit growing.

Zootechnicians are very interested in topics related to the protection of arable crops and vineyards. The greatest interest of zootechnicians in agronomic topics is explained by the little knowledge they have about these particular topics.

We notice that there are differences between the two groups of extensionists, according to the experience of working in extension, in terms of training they need on zootechnical and agronomic issues. Less experienced extensionists consider less important topics for irrigation, fertilization, dry grass preparation and silage compared to more experienced extensionists. It can be understood that these specialists think that they deal mainly with advising farms with crop production and fruit growing.

For technical topics of agriculture and livestock, the majority (80.5%) of the group of less experienced extensionists call them important and very important, compared to the group of experienced extensionists, they rate them at 76%.

But what was not expected is that the group of less experienced extensionists considers the topics related to communication and extension important and very important to the extent of 73.4% against 78.1% of the group with more experience.

In terms of the most suitable time for training, most extensionists are responded for spring (59.1%).

Regarding the purpose of the extensionists to participate in the trainings, the three main answers are: (i) “To get acquainted with new technologies and to pass them on to the farmer”; (ii) “Training on technical issues and updating knowledge” and (iii) “To communicate as freely as possible with farmers and contact groups”.

ACKNOWLEDGMENTS

The authors of this paper are thankful to the extensionists of AREB -Tirane and AREB-Korçe for there contributions in filling the questionnaires in relation with their training needs. In addition, we are thankful to Prof.Asc Edvin Zhllim and Prof.Asc Drini Imami for the very good discussions for the training needs of extensionists.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

METHODS AND TECHNOLOGIES USED TO INCREASE THE PROLIFICACY OF LOCAL SHEEP BREEDS

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Abstract

The orientation of the breeding and exploitation of sheep for meat production worldwide, imposed a basic technological element, namely obtaining as many lambs as possible, which is the most important goal in increasing this production. The increase of the reproduction indices creates the premise of the profitability of the sheep regardless of the exploitation system practiced. The interest is channeled especially for the increase of fecundity, fertility, and especially prolificacy indices. The intensification of prolificacy is a major objective in the exploitation of all breeds of sheep because it leads to the numerical increase of livestock and meat production. Twin lambs have intense growth energy, which allows the weight of the simple ones to be equalized until the age of the first shearing, and the expenses occasioned by the maintenance of the second lamb are generally reduced. Most of the sheep breeds that are raised in our country are characterized by fairly low values of prolificacy (105-110%), the highest value being recorded in the Merinos de Palas breed. Prolificacy is dependent on genetic factors, especially on the breed. Reproductive characters are characterized, unfortunately, by a low value of the heritability coefficient (h^2 = 0.05-0.25), which is determined mainly by non-additive genes, so the selection for this type of characters is very difficult and long-term, the fastest way to improve prolificacy in local sheep breeds is by crossing them with rams from prolific breeds. Internally, within the I.C.D.C.O.C - Palas Constanta, the Prolific Line - Palas was created, following the crossing of Merino de Palas sheep with rams from Romanov, Friesian, and Finnish Landrace breeds, which are characterized by an average prolificacy of 160-180%. Prolific breeds can be used in simple industrial crossings to increase meat production (females prolific breeds x males meat breeds), or to obtain F1 hybrid prolific females in the year I (local breed females x males prolific breeds), which in the second year are crossed with males from specialized meat breeds (double or triple industrial crossings).

Key words*: crossbreeding, local breeds, meat breeds, prolificacy, reproduction indices, selection.*

INTRODUCTION

Sheep breeding and exploitation is an ancient activity, with great traditions in Romania, it is a basic branch of animal husbandry that has developed in different pedoclimatic areas, depending on the biological characteristics of the breeds exploited and market requirements (Ștefănescu et al., 1973).

At the national level, the reconsideration of the directions of sheep exploitation and the orientation of the activity of breeding this species on the principles of the market economy stimulated the preoccupations for the increase of milk and meat productions. In this context, even if wool production is no longer a priority in the current conditions, by ensuring a quantitative level, but especially its quality in the breeds and populations specialized for milk and meat can obviously ensure an additional income of farmers and capitalization efficient use of this textile raw material with special physical-mechanical, technological and hygienic properties (Taftă, 1997).

The concerns of sheep breeders must be directed towards increasing and improving the quality of meat in sheep and for increasing the quantity of milk, this can be achieved by creating breeds, populations and lines of specialized sheep for meat or milk production, without neglecting wool production, simultaneously with the application of selection to existing breeds in Romania and by improving growth and exploitation technologies (Pădeanu & Voia, 2010).

Establishing the relationship between the different productions and the limits between which they can increase, without prejudicing the physiological balance, is the problem of major practical significance, in order to increase the profitability in the field of sheep breeding. There is no physiological antagonism between wool production, meat production and milk production, but on the contrary a reduced positive phenotypic correlation, however, even under very good care and feeding conditions, wool production, meat production and milk production can't be raised in parallel, indefinitely (Taftă, 2008).

Sheep farming has been and remains an important goal, as this species can use less used feed as food and housing and care needs are less expensive than other species (Călin, 2003).

Assisted reproduction, shortening the calving-breeding period, deseasonalization of heat and calving and accelerating calving by non-hormonal and hormonal methods, can successfully contribute to both calving per year, three calving in two years, and increasing the prolificity of sheep (Răducuță, 2000).

The success of theapplication of these biotechnological methods will be possible only where there is a sustained concern for the correct application of all technical and biotechnological stages that can be achieved by obtaining outstanding results in increasing the birth and prolificity of sheep and obtaining offspring with superior morphoproductive traits. to justify the effort to disseminate knowledge to the breeder on the introduction and expansion of biotechnological methods of reproduction.

The problem of intensifying reproduction in sheep by reducing the interval between calvings is a concern both nationally and globally (Dărăban, 2016).

The reproductive capacity of sheep is one of the main factors that determine the multiplication and improvement of livestock, which is why we consider that the chosen topic is current.

Lately, the growing needs for products of animal origin and especially food have naturally led to a reconsideration of the old systems of breeding and exploitation of animals, including sheep, being replaced by industrial systems.

As already shown, in recent years the breeding of sheep in our country has acquired new guidelines that give sheep production a much more pronounced character of intensity than it had in the classic system of breeding and exploitation.

Currently, sheep farming is increasingly oriented towards meat production, which will become the main production in some areas of the country in the near future. Increasing the production of sheep meat and increasing the economic efficiency of this activity are largely conditioned by the intensification of the breeding process.

The orientation of the breeding and exploitation of sheep for meat production worldwide, imposed a basic technological element, namely the intensification of the breeding process, as obtaining as many lambs as possible, is the most important goal in increasing this production (Taftă, 1983).

Reproduction intensification includes a series of measures and methods whose main purpose is to transform seasonal polycyclicity into annual polycyclicity, facilitating the installation of gestation, including in the anestrus phase, as well as advancing the age of the first mount from 18 months to approx. 8-10 months, which will allow to increase the prolificacy, the possibility of organizing calvings throughout the year and obtaining a number of 1-2 lambs every 7-8 months (Răducuță, 2000).

Research conducted worldwide and nationally in the field of sheep breeding has shown that the reproductive function can be modified and directed to advance estrus and even the manifestation of heat throughout the year, within physiological limits, provided certain feeding conditions and maintenance (Taftă et al., 1997).

The study of the physiological characteristics of reproduction showed that the process of reproduction in animals is suitable for a scientific direction and regulation, the sheep being - among the domestic species - the most receptive to the stimulation of sexual activity.

MATERIALS AND METHODS

In order to create this material, the following bibliographic materials were studied: specialized books on the reproduction of domestic

animals or sheep breeding, represented either by unique textbooks specific to the profile faculties in our country, or by specialized textbooks, specialized brochures, specialized courses, papers presented at various national and international symposia.
The methodology of the paper consisted in presenting the prolificacy index (definition, calculation relationship and influencing factors), presenting the methods and technologies used so far to increase prolificacy in sheep in our country, along with the results obtained in scientific research by profile, and finally the presentation of the conclusions arising from the researched material.

RESULTS AND DISCUSSIONS

The *prolificacy* in sheep varies within quite wide limits, being determined by internal and external factors (breed, age, breeding season, food, hormonal substances, etc.). In terms of prolificacy, sheep are located between uniparous and multiparous species.
Prolificacy is calculated by relationship (Paraschivescu, 1969):

$$P\% = \frac{100*m}{f}$$

In wich:
p = prolificacy;
m = number of lambs;
f = number of births.

In some sheep breeds, the prolificacy is very high, even constituting a breed character (Finnish Landrace, Romanov, Oldenburg, Friesland, Border-Leicester, Chios, Hu-Yang, etc.).
The percentage of prolificacy obtained in the sheep from the resorts of the former Institute of Zootechnical Research is shown in Table1.

Table 1. Fertility and prolificity of local breeds (Taftă, 1983; 1997)

Race	Fecundity (%)	Prolificacy (%)
Merinos de Palas	93	110-146
Merionos de Transylvania	93	112-120
Spancă	95	110-116
Țigaie	97	104-107
Țurcană	98	103-105
Karakul	97	105-108

Prolificity is dependent on genetic factors, but is also influenced by environmental conditions, may have its own innate character or can be acquired through various biological or genetic interventions.
In order to increase the prolificacy, in our sheep farms the selection of breeders from twin calvings, the preparation of sheep and rams for breeding, their rational feeding during the breeding period as well as the pregnant sheep, the hormonal method, the crossing with prolific breeds to enrich the gene pool are applied. with the valuable genes of these breeds, especially for obtaining lambs for fattening.

Factors influencing prolificacy. Prolificacy is dependent on several genetic factors (breed, population and individual) and the environment (diet, hormonal treatments, age of sheep, weight of sheep, breeding season), which can be controlled by sheep breeders (Bogdan et al., 1984).
The breed has a major influence, as there are highly specialized breeds in the direction of prolificacy such as: Bluefaced Leicester 180-200%, Frisian 200-225%, British Sheep Dairy 220-300%, Romanov 250-300%, Hu Yank 200-300%, Finnish Landrace 300%. Specialized breeds from other countries are more than twice as prolific as indigenous sheep breeds (Răducuță et Tăpăloagă, 2010).
In Romanov, the reproductive character represented by fertility and prolificacy is higher than 250%, while in other breeds (Țurcană, Țigaie, Karakul de Botoșani) the prolificacy tends to be very close to 110% (Pascal, 2015).
Population and individual. Within each race there are populations and within them, individuals with a much greater prolificacy. The selection is based on individual variability in order to be able to achieve populations with high prolificacy (Pădeanu, 2012).
Food. Feeds rich in essential amino acids, energy, vitamins and minerals and with a moderate content of phytoestrogens cause the simultaneous maturation of 2-5 ovarian follicles. In order to obtain a high prolificacy, it is necessary to maintain the level of stimulating nutrition both during the breeding campaign and in the first month of gestation, in order to avoid embryonic losses. In the autumn season, during the period of preparation for breeding

and breeding, as well as in the first month of gestation, it is recommended to graze on green stubble (clover, alfalfa, pastures grown in a mixture of legumes and grasses, peas, oats, wheat, barley) or on meadows poisoned alternately with pastures rich in carbohydrates (corn stubble) (Pădeanu, 2012; Stoica, 1994; 1997). Through these measures a prolificacy can be achieved at least at the upper limit of the breed.

The age of the sheep significantly influences the prolificacy, being lower in sheep, then it increases until the age of 5-6 years, when it is maximum, after which it decreases with advancing age. In the growing female youth, a large part of the nutrients is still directed towards the development of the body, and in the elderly sheep the efficiency of digestion and metabolism decreases in parallel with the increasing incidence of gynecological diseases. In relation to age, research conducted at ICPCOC - Palas established that the prolificacy is lower in primiparous sheep, increases up to 5-6 years (137-140%), then gradually decreases to 116.6% at the age of 8 years (Scheul et Petcu, 1975; Sandu, 1993).

The body weight of the sheep shows a moderate influence on the prolificacy, in the sense that the heavier sheep perform a significantly higher number of multiple calvings compared to the sheep with lower weight. Body weight positively influences the prolificacy, finding that Merino de Palas sheep with a body weight between 66-80 kg, register the highest value, respectively 131-139%, while sheep with a weight of 61-65 kg, have a prolificacy of only 126% (Bogdan et al., 1984; Taftă, 1983). However, the improvement of sheep for prolificacy does not go in the direction of increasing body weight, as this would entail additional costs with the maintenance of those sheep.

The breeding season has an indirect influence on prolificacy through the level of feeding, the length of daylight and the intensity of light.

Frequently, the highest prolificacy is achieved in sheep breeds in our country in the autumn season, when sheep benefit from abundant forage, moderate brightness (1: 1). After the breeding done in the first part of the breeding season, the most multiple births are obtained for the native sheep breeds (Pădeanu, 2012).

Methods and technologies used to increase prolificacy. To increase prolificacy, in our sheep farms we apply the selection, preparation of sheep and rams for breeding and rational feeding during the breeding period as well as pregnant sheep, hormonal method, crossing with prolific breeds to enrich the gene pool with the valuable genes of these breeds, especially for obtaining lambs for fattening.

Selection to increase prolificacy. The ability to give birth to several lambs at birth varies between breeds and between individuals, due to the different number of eggs which is dependent on the special concentration of blood in gonado stimulating hormones. (Ștefănescu et al., 1973).

Reproductive characters are unfortunately characterized by a low value of the heritability coefficient (h^2 = 0.05-0.25), being determined mainly by non-additive genes, so the selection for this type of characters is very difficult and long duration, the fastest breeding route being the breeding of rams of the prolific breeds (Mochnacs et al., 1978).

It should be noted, however, that in the hereditary transmission of prolificacy females participate with about 3/4, respectively in a proportion of 75%, while males only with ¼ (Taftă, 1983).

Also, the existence of differences by race, lineage and family in terms of multiple calvings, is a guarantee that increased genetic proliferation and birth rate is possible and that the selection made for such characters will get an answer at an acceptable rate, despite the coefficients of reduced heritability, if the choice of individuals to produce the next generation is made after several production cycles, and the rams are tested for offspring and collaterals (Bradford et al., 1981).

Retention for hatching of males and females from twin births can be a great way to identify future mothers and rams that have a favorable genetic potential for increasing fertility and prolificacy (Taftă, 2008).

At the same time, the practice of an indirect selection by characters associated with prolificacy, such as body weight (Table 2), milk production (rG = 0.13-0.16), skin reserves, degree of coverage of the face with wool, rate of ovulation and scrotal

circumference, can lead to encouraging results (Bodin et al., 1999).

Table 2. Genetic correlation between prolificacy and weight at different ages (Analla et al., 1995)

Genetic correlation	Prolificity - birth weight	Prolificity - weaning weight	Prolificity - weight at 90 days	Prolificity- weight at 180 days
rG	0.18	0.48	0.36	0.22

However, the selection and breeding of sheep with a genetically enhanced character for twin births can be used as one of the ways to raise prolificacy, especially in our country where prolific breeds are lacking.

Food. Another method of influencing prolificacy has been shown to be nutrition. Feeding experiments have shown that sheep have in their genofond the capacity to produce a greater number of lambs, a genetic possibility that can be enhanced by the use of favorable environmental factors, especially through adequate feeding of sheep in the breeding season. reproduction and during pregnancy. Selection associated with optimal feeding and care conditions can be used to increase prolificacy. In 1970, at the I.C.D.C.O.C - Palas, a group of Merino sheep with double and triple births began to be selected, which were mated with rams from twin births, in order to create a prolific line (Florea, 2018).

The quality and level of the ration administered to the sheep in the period before the start of the breeding season is also of considerable importance. Research in this regard has shown that if at that stage, feeding is done using balanced rations, after breeding will be obtained not only an increase in the number and weight of lambs born but also a higher percentage of twin births, so stimulant feeding. it also induces an increase in prolificacy (Pascal, 2015). This stimulating feeding procedure is known as flushing. Particular attention should also be paid to the mineral supplement and vitamins.

The supplementation of the ration applied during the preparation period for breeding, therefore leads to obtaining beneficial effects in increasing the prolificacy. Following the research, it was found that the application of this method (flushing) for a period of only two weeks, increases the number of twin births from 6-8% to approx. 20%, and when applied for 4-5 weeks the twin births reaches to 40% (Tafta et al., 1997). In general, a good preparation of sheep for reproduction on the basis of green mass, in sufficient quantity and of good quality, leads to higher fertility and prolificacy.

Crossing. The main method of increasing prolificacy in sheep is cross-breeding with rams of the prolific breeds, which is widely applied in many countries (Russia, USA, New Zealand, England), in order to produce an increased number of lambs for fattening (Ștefănescu et al., 1973).

In our country, a series of experiences were organized within the I.C.D.C.O.C - Palas Constanța for crossing local sheep with rams of prolific breeds, which highlighted the fact that the F_1 crossbreeds obtained had a much higher prolificacy than the local breeds (Table 3).

Table 3. The effect of sheep hybridization for prolificacy (Ionescu et al., 1985)

Sheep breeds	Average prolificacy, %	
	Maternal breed	Metis F_1
Romanov x Merinos de Palas	127.3	196.9
Romanov x Spanca	135.7	170.3
Romanov x Turcana	102.9	164.7
Finnish Landrace x Merinos de Palas	127.3	163.3
Finnish Landrace x Spanca	135.7	170.7
Finnish Landrace x Tigaie	111.3	180.0

In general, the purpose of crossing sheep of less prolific breeds with rams of breeds that have this pronounced trait is to obtain F_1 females with hereditary substrate enriched with genes for prolificacy. Crossbred females are then crossed with rams of the meat breeds to obtain an increased number of lambs for meat.

In France, Romanov is preferred as a prolific breed due to its high prolificacy (250%), long breeding season (8 months/year), excellent fertility, strong maternal behavior and good product viability, while in England Finnish Landrace and Border-Leicester breeds are preferred, approx. 35% of the sheep livestocks in these countries are bred by crossbreeding (Bonnes et al., 1991).

In our country were imported rams of the Border-Leicester breed, characterized by a good prolificacy. These rams have been crossed

with sheep of our breeds and types to produce prolific mixed breed sheep to be crossed with rams of meat breeds (Ile de France, Suffolk). Such an experiment was carried out at I.C.D.C.O.C - Palas, where 84 F1 ewe lambs (Border Leicester x Spanca) were crossed during 1972 with meat rams (Florea, 2018).

Lately, the researchers attention is directed towards the creation of synthetic (composite) populations, resulting from the crossing of 3-4 breeds, thus combining the prolificacy of some breeds recognized in this respect, with breeds that have an extended reproduction duration on the whole year (e.g. 50% Finnish Landrace + 25% Dorsethorn + 25% Rambouillet), or with breeds that have a high growth intensity (e.g. 50% Finnish Landrace + 25% Suffolk + 25% Targhee), which creates the possibility of maximizing the intensity of selection and maintenance of heterosis at high values for several generations (Taftă et al., 1997).

Internally, within the I.C.D.C.O.C Palas, the Prolific Line - Palas was created, following the crossing of Merino sheep with rams of the Romanov and Finnish Landrace breeds, which is characterized by an average prolificacy of 160-180% (Taftă, 1998).

Recently, in the ADER 5.1.2. Project, good results regarding prolificacy were obtained for local sheep breeds by crossing them with the prolific breeds (Romanov x Merionos de Palas), obtaining in F1 hybrid sheep an average prolificacy of about 160%, compared to the Merinos de Palas breed where the prolificacy was 105% (MADR - Proiect ADER 5.1.2).

Increased prolificacy can also be achieved by the introgression of a major gene (F/F and F/+ fertility gene), as is the case of the Booroola, Galway, Toka, Olkuska genes, which can increase the frequency of twin births in a non-proliferative population, being in fact among the latest achievements of science in this field (Bodin et al.,1999).

Hormonal method. This involves the use of estrogen hormones such as S.I.G. to increase the number of lambs at birth, using at the same time with the synchronization of estrus**.**

Hormone treatment aims to provoke twin births or stimulate the appearance of estrus in the off-season to obtain two births in one year or three births in two years. Regarding the first aspect, some of the results obtained in our country and in other countries, as well as the prospects of application in large production, were previously shown. Thus, it was concluded that the challenge of poliovulation has more chances of application in production, in association with the synchronization of estrus in the normal breeding season. The orientation of sheep breeding towards meat production has also raised in our country, lately, the problem of synchronous induction of estrus in the off-season and scientific research indicates convenient solutions in this regard (Ștefănescu et al., 1973).

Numerous researches have been carried out both globally and in our country, using as sexually active substances serum gonadotrophins in the form of S.I.G., alone or in combination with progesterone, as well as similar synthetic preparations, administered in different ways: per os, subcutaneous injections, intravaginal weights, and more recently in the form of implants.

Timariu et al. (1961), using progesterone injections and S.I.G. in the months of April-May in Merino de Palas ewes who gave birth in January obtained a fecundity of 68% and a prolificacy of 140%.

Hormone treatments are an effective method, available to sheep breeders. In most cases, hormonal treatments for heat synchronization also aim to increase prolificacy, so the dose of PMSG will increase, in this case, by about 20-30% (compared to 500 IU PMSG, 600-700 IU PMSG is inoculated). At present, in France, most sheep farms for milk and meat undergo hormonal treatments, which aim to synchronize heat and increase prolificacy (Pădeanu, 2012).

From the conclusions drawn from these experiments, it emerged that in sheep breeding there are great possibilities to increase the prolificacy by the hormonal method, but the scope is still limited in our sheep farms, being related to its laborious nature.

In addition, hormonal treatment involves the early and very early weaning of lambs, especially when it comes to producing two births a year. Feeding these lambs with milk substitutes or with proper starters and starters also raises a number of issues in terms of ease of application in large production.

From the above, it results that in sheep breeding there are great possibilities to

intensify reproduction both by using genetic methods and by directing external factors.

CONCLUSIONS

From the presented material, it appears that the prolificacy of local sheep breeds is still at a very low level. Over time, a number of methods have been used in our country to increase the prolificacy of local sheep breeds.
Direct or indirect selection for prolificacy associated with optimal breeding and care conditions can be a way to increase prolificacy. The main and fastest method of increasing prolificacy, however, is the crossing of local breeds of sheep with prolific rams.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

STUDY ON ANIMAL BREEDING IN THE MOUNTAIN AREA OF ROMANIA IN RELATION TO THE DISTRIBUTION OF COLLECTION CENTERS AND MILK PROCESSING UNITS

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Abstract

The paper aims to present the situation of animal husbandry activity in the counties with mountain area in Romania, in 2019, in relation to the activity of milk collection and processing in the 27 counties with mountain area. This paper is based on statistical data provided by the National Sanitary-Veterinary and Food Safety Authority, the National Institute of Statistics and the National Agency for Mountain Area (ANZM). These data were statistically processed within the National Agency for Mountain Area in the following indicators: number of counties with mountain area, number of administrative-territorial units in the mountain area, total area of the mountain area (km^2), number of animals in the mountain area (cattle, sheep, goats) in 2019, the total area of permanent meadows in the mountain area (ha), number of milk collection centers in the counties with mountain area, number of milk processing units in the counties with mountain area. The statistically processed data show that in counties with large herds of animals there are milk processing centers and units compared to counties with small herds of animals that do not have centers and units of milk processing or are few.

Key words*: animal husbandry, milk colection center, milk processing units.*

INTRODUCTION

The disadvantaged mountainous area of Romania is a special territory of national interest, with a huge economic, social, cultural and environmental potential. The strategic guidelines present the main directions to ensure the increase of attractiveness and sustainable development of the disadvantaged mountain area, by valuing resources, stabilizing the population, maintaining cultural identity, increasing economic power at local level, while maintaining ecological balance and protecting the environment. By achieving the established objectives, the sustainable economic, social and environmental development of the disadvantaged mountain area will be achieved by protecting and responsibly capitalizing on mountain resources, taking into account the effects of climate change, preventing depopulation of these areas and degradation of traditions, occupations and cultural specificity. theirs. Ensuring the means for the balanced development of mountain resources, on par with other areas in Romania and the EU, in terms of income and living conditions, must benefit from intense preventive and effective support from the state. Mountain areas must benefit from a specific policy defined according to the principles of sustainable development, which ensures the needs of the present without compromising the chances of future generations. The strategic guidelines also aim to reduce the imbalance between the more favored and disadvantaged mountain regions, marked by permanent natural constraints, targeting the whole economic problem, social, cultural and environmental issues. Mountain policies must facilitate inter-municipal and inter-regional cooperation within the national framework, cross-border and trans-national cooperation (National Strategic Guidelines for the Sustainable Development of the Disadvantaged Mountain Area, 2014-2020).

MATERIALS AND METHODS

In order to characterize the situation of the mountain area in Romania, the following indicators were used: number of counties with

mountain area, number of Administrative-Territorial Units in the mountain area, total area of the mountain area (km^2), number of animals in the mountain area (cattle, sheep , goats, Statistical Yearbook of Romania 2019, http://www.insse.ro), number of milk collection centers in mountain counties, number of milk processing units in mountain counties (ANSVSA-2019). The analyzed period was 2019, and the data were provided by the National Sanitary-Veterinary Authority and for Food Safety and also by the National Institute of Statistics. The primary data were systematized, processed and interpreted by methods specific to such research (, ± s, s, V%, p significance test, confidence interval). It is recommended that the estimation of a theoretical parameter be done by means of an interval not of a single value. This interval is called the confidence interval. The estimated parameter most likely belongs to the confidence interval. A string of values of an estimator of interest calculated so that for a chosen error probability to include the true values of the variable. The range defined by the critical values will include the population estimator with a probability of 1-α. Also, the data analysis was done in terms of merging and correlating with numerous field observations. These data were processed within the National Agency for Mountain Area.

RESULTS AND DISCUSSIONS

The mountainous area of Romania (Figure 1) has a total area of 71,381.48 km^2, and includes 658 Territorial Administrative Units, respectively 27 counties with mountainous area (Table 1), which represents 30% of the country's territory, of which 577 communes, 81 cities and municipalities and 3536 villages. The surface of permanent meadows in the counties with mountainous area is 4,154,663 ha. The herds of animals from the mountainous area of Romania are presented in Table 1.

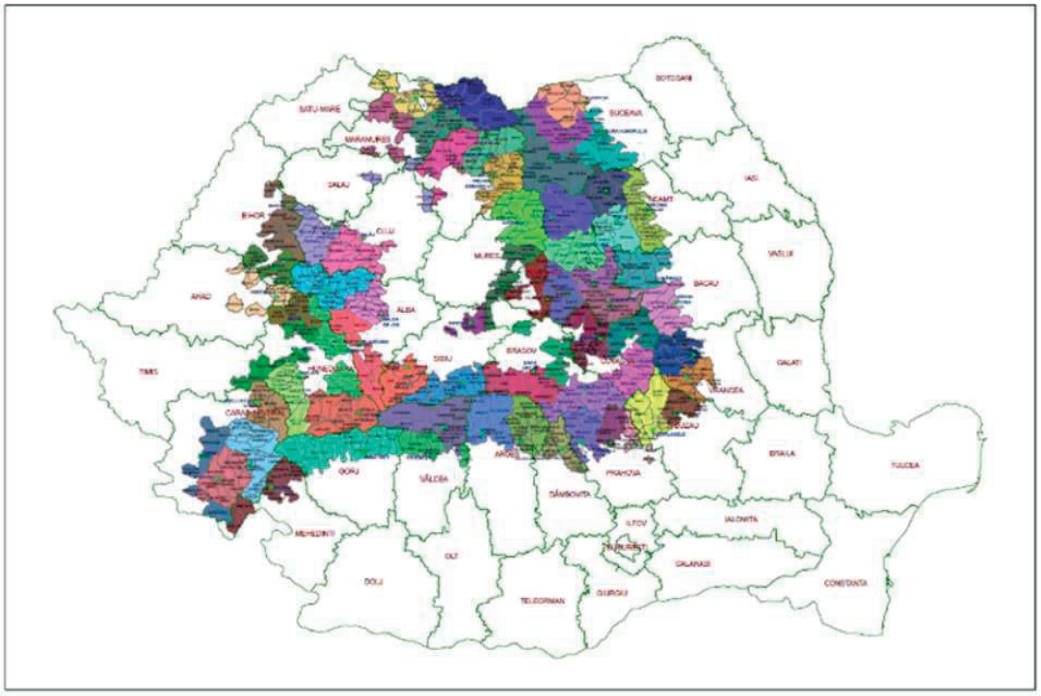

Figure 1. Mountain area of Romania

Table 1. The situation of the livestock, of the surface of permanent meadows and of the milk collection and processing center at the level of the counties with mountainous area in Romania (ANSVSA - 2019 source)

No. crt.	Mountainous county	Cattle	Sheep	Goats	Permanent meadows	Milk collection centers	Milk processing units
1.	ALBA	94,449	506,724	32,247	187,990	27	7
2.	ARAD	54,043	725,057	26,166	135,077	2	8
3.	ARGEŞ	58,686	218,201	35,720	146,898	2	4
4.	BACĂU	58,544	223,004	86,986	125,826		2
5.	BIHOR	73,357	443,381	26,841	172,209	11	4
6.	BISTRIŢA-NĂSĂUD	76,034	414,058	29,622	190,212	75	6
7.	BRAŞOV	66,365	545,894	18,509	159,384	9	6
8.	BUZĂU	52,781	290,921	54,474	119,930	1	3
9.	CARAŞ-SEVERIN	29,022	314,897	16,181	255,741		1
10.	CLUJ	63,278	607,516	28,564	245,482	52	9
11.	COVASNA	46,675	238,422	9,259	102,196	71	2
12.	DÂMBOVIŢA	31,038	67,316	32,980	62,503	2	3
13.	GORJ	45,908	141,496	37,045	128,897	2	2
14.	HARGHITA	89,602	245,556	21,960	316,124	67	10
15.	HUNEDOARA	42,925	294,505	23,155	201,217	1	3
16.	MARAMUREŞ	85,523	278,744	32,871	217,907	60	7
17.	MEHEDINŢI	30,379	135,826	80,438	92,764		1
18.	MUREŞ	78,285	504,200	38,213	183,519	86	12
19.	NEAMŢ	71,565	214,236	40,277	110,428	13	3
20.	PRAHOVA	43,866	253,400	50,444	108,395		3
21.	SĂLAJ	26,008	337,132	21,251	110,999	16	
22.	SATU MARE	44,149	260,586	15,986	75,649	12	5
23.	SIBIU	52,885	658,153	32,118	177,235	12	3
24.	SUCEAVA	128,038	256,875	19,474	164,691	113	16
25.	TIMIŞ	43,227	795,943	22,725	147,303	19	4
26.	VÂLCEA	42,320	120,588	28,113	139,425		1
27.	VRANCEA	46,078	170,754	43,939	7,6662		2
28.	**TOTAL**	**1,575,030**	**9,263,385**	**905,558**	**4,154,663**	**653**	**127**

In Table 1 we can see that the number of animals in the counties with mountainous area varies from one county to another, the largest number of cattle is registered in Suceava county 128,038 heads, followed by Alba county, with 94,449 heads, Harghita with 89,602 heads, Maramureş with 85,523 heads, Mureş with 78,285 heads, Bistriţa-Năsăud with 76,034 heads. It should be mentioned that Suceava County occupies the first place in cattle
herds for a long time and the explanation lies in the fact that the species has productions such as milk and meat, which are capitalized in the form of mountain products, being herbivorous capitalizes very well feed in the mountains. Of course, the largest flocks of sheep and goats are found in counties with tradition and a special form of relief that favors the exploitation of these species. Therefore sheep herds in the counties with mountain area it is presented as follows: Timiş county with 795,943 heads, Arad with 725,057 heads, Sibiu with 658,153 heads, Cluj with 607,516 heads, Alba with 506,724 heads, Mureş with 504,200 heads. Regarding the goat herds in the counties with mountain area, the highest number was registered in Bacău county with 86,986 heads,

Mehedinți with 80,438 heads, Buzău with 54,474 heads and Prahova with 50,444 heads. The herds of animals are positively correlated with the surface of permanent meadows in the counties with mountainous area. The largest areas of permanent meadows are found in Harghita County (316,124 ha) (Figure 2). Table 1 shows that the counties with mountain areas in Romania that have the largest herds of cattle have the most collection centers and milk processing units such as: Suceava with 113 milk collection centers (Figure 3) and 16 processing units (Figure 4), Mureș with 86 collection centers and 12 milk processing units, Bistrița-Năsăud with 75 collection centers and 6 milk processing units, Harghita with 67 collection centers and 10 milk processing centers, Cluj with 52 collection centers and 9 milk processing units, Alba with 27 collection centers and 7 milk processing units. The positive correlation between cattle herds and the number of collection centers and milk processing units is explained by the fact that the bovine species has a monopoly on milk production, providing over 93% of world and national milk production. Therefore, milk must be processed and recovered in terms of economic efficiency. A general expression of economic efficiency is given by the relationship between the useful effects obtained from a certain economic activity and the expenses, ie the efforts made from that activity. The fewest milk collection centers and processing units are located in Bacău, Caraș-Severin, Dâmbovița, Gorj, Mehedinți, Vâlcea, Vrancea, because the number of animals in these regions is low. In Neamț County, there was a decrease in cattle herd of approximately 15% during the years 2010-2018 owned by the population in the area. This reduction was determined by: the extremely small size of the farms (1-2 heads) and the massive slaughter of animals (Nistor-Anton & Maciuc, 2019). The milk law project aims to regulate the marketing of dairy products, to increase consumer confidence in domestic dairy products and to eliminate falsified products (Coman et al., 2019).

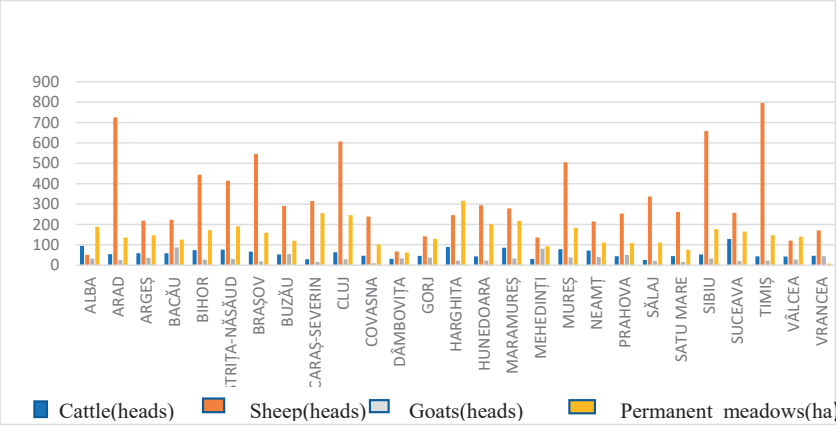

Figure 2 .The situation of the herds of animals and of the surface of permanent meadows in the counties with mountainous area from Romania

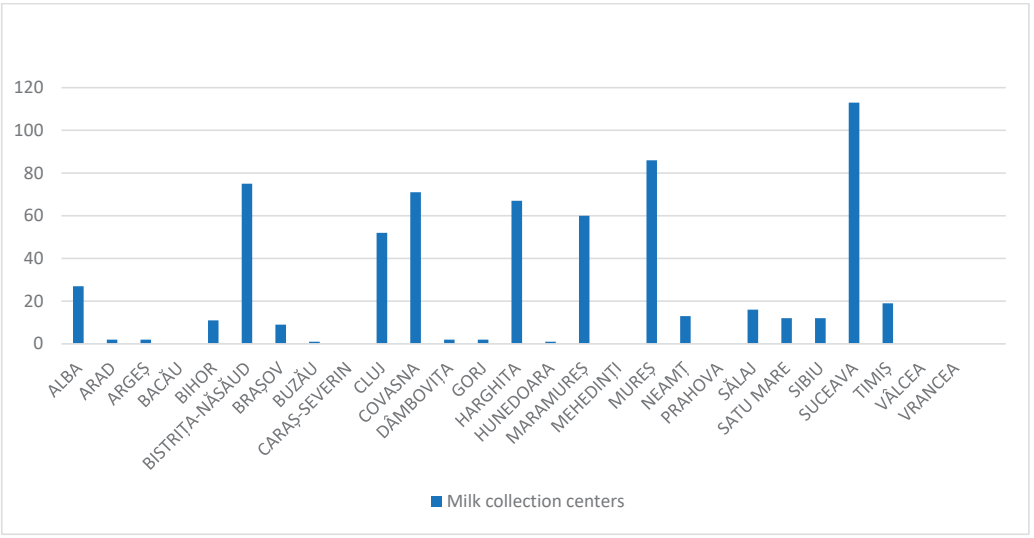

Figure 3. Distribution of milk collection centers in the mountainous area of Romania

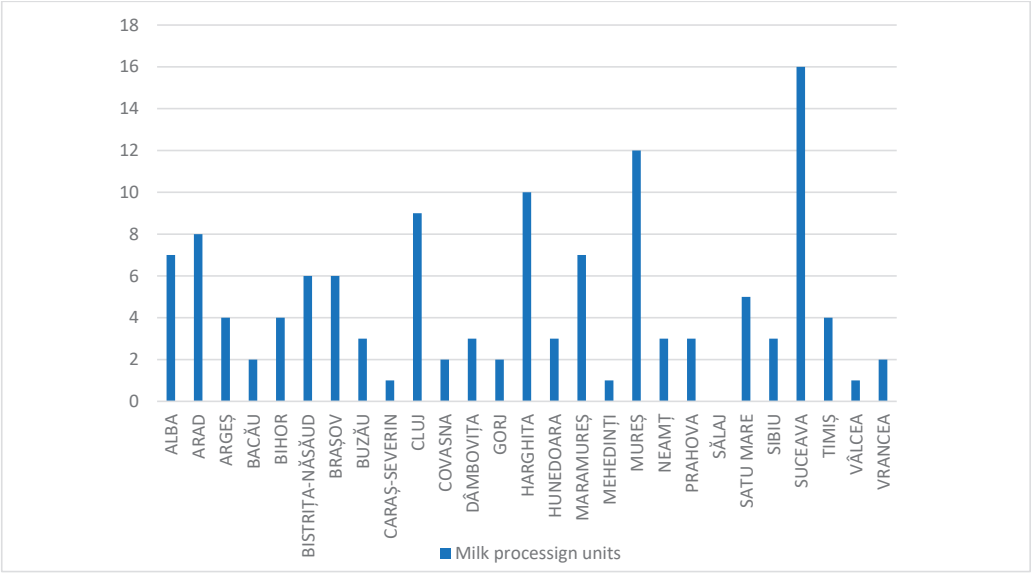

Figure 4. Distribution of milk processing units in the mountainous area of Romania

CONCLUSIONS

From the study, the following conclusions can be drawn:

1. The largest herds of cattle were registered in Suceava County, in sheep in Timiș, Arad and Sibiu counties, and in goats in Bacău, Mehedinţi, Buzău and Prahova counties.

2. The positive correlation between bovine herds and the number of collection centers and milk processing units is explained by the fact that the bovine species has a monopoly on milk production, providing over 93% of world and national milk production. Therefore, this milk must be processed and used in conditions of economic efficiency.

3. The herds of animals are positively correlated with the surface of permanent meadows in the counties with mountainous area. The largest areas of permanent meadows are found in Harghita County (316,124 ha).
4. The predominant increase of cattle in the mentioned counties, with each passing day, becomes more and more difficult due to the fact that the population is aging, the young population has migrated to cities or even abroad, the price of milk is very low (0.60-0.85 lei/liter), being brought to the milk processing units large quantities of milk from outside Romania at lower prices. Mountain farms have small areas, farmers not being able to negotiate the price of milk with processors.
5. We want to organize farmers in cooperatives, producer groups in order to sell at a better price the milk obtained from animals, a better price for animals delivered to the slaughterhouse, the use of farm products in the form of "mountain product". Accordingly, mountain farmers must be supported by government programs to encourage young farmers to grant, subsidizing the price of milk and meat, to ensure the continuity of this animal husbandry activity in the mountainous area of Romania, but also to avoid degradation of areas of permanent meadows in the mountain area.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

RESEARCH REGARDING THE EFFECT OF THE NUMBER OF MILKINGS A DAY ON MILK PRODUCTION AT PRIMIPAROUS COWS

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Abstract

After the first 7 days, the primiparous cows milked three times a day achieved 20.00 kg milk postpartum, corrected at 3.5% fat, while the cows milked twice a day achieved an average production of 17.10 kg milk postpartum, corrected at 3.5% fat. There is an additional quantity of 2.9 kg milk at the primiparous cows milked three times a day (an increase of 16.9%). Statistically analysed, this increase is distinctly significant (P<0.01). After 21 days of experiments, the average milk production of the primiparous cows milked twice a day reaches 19.15 kg milk with 3.5% fat, and the average milk production of the ones milked three times a day reaches 20.50 kg milk with 3.5% fat; for the latter case, there is a decrease of production of 3.8% compared to the previous lapse of time, probably due to certain changes of the physiological status. After 28 days of experiments, the average production of the primiparous cows milked three times a day reached 22.80 kg milk with 3.55 fat, and the average production of the ones milked twice a day reached 18.85 kg milk with 3.5% fat, registering thus a difference of 2.95 kg milk (14.8%). Statistically analysed, this difference is distinctly significant (P<0.01).

Key words*: fat, milk corrected, primiparous.*

INTRODUCTION

Cows are generally milked twice (2x) a day. With the introduction of milking performed by milking robots it is necessary to know the productive effect of more than two milkings per day. Passing to more milkings a day often increases milk production by 10-20% (Erdman & Voiner, 1995; Rastani et al., 2007).

Although the udder is full and the milk pressure is high, milking is not possible, or it can only be done partially. For milking to be possible, the presence of a hormone, called oxytocin, is needed, which causes the contractile elements surrounding the alveoli to contract and the walls of the collecting ducts and the nipple sphincter muscle to relax. Under the action of oxytocin, the milk accumulated in the lumen of the alveoli is pressed to the milk tank from where it can be evacuated by milking.

Oxytocin is produced under the action of stimuli caused by the presence of the milkman, by the specific noises of milking, but the most important stimuli are washing with warm water, wiping and preparatory massage of the udder performed by the milkman. Under the action of stimuli, the secretion of oxytocin in the brain begins immediately, from where it reaches the udder through the blood, where it manifests effect in less than a minute.

It should be noted that of the total milk secreted between two milks only a part is stored in the milk tank, and the larger amount is accumulated in the alveoli and canals, an amount that can be extracted only under the action of oxytocin (Bar-Peled et al., 1995).

The effect of oxytocin is about 6-8 minutes, after which it disappears from the blood. Milking must be carried out during this time, because in the absence of oxytocin the milk can no longer be evacuated from the alveoli. The release of milk also stops when disturbances occur during milking, for example: hitting the cow, foreign noises, etc., which cause the appearance of another hormone in the body, adrenaline, which immediately annihilates the effect of oxytocin. The aim is to ensure normal conditions, without disturbances, and with the preparation of the animal for milking and the preparatory massage to ensure the normal secretion of oxytocin (Dahl et al., 2004)

The fact that the cow "does not give milk" in certain situations has a physiological explanation, and the culprit in most cases is the milkman and the stressors that occur during milking. The action of oxytocin has also been

demonstrated by experiments, when oxytocin was injected into a herd of cows immediately after the end of milking and milk quantities of between 0.5 and over 1 liter per cow could be milked. In reality we not talk about a complete milking, because in the udder there is always a quantity of milk, which is called residual milk, but this amount should not exceed 5-10% of the milk milk. A large amount of residue does not only mean economic loss, due to the high percentage of fat, but also an obstacle in the process of milk secretion, because the udder fills faster, increasing milk pressure, the secretion stops and the cow no longer produces, milking cannot be a routine activity that anyone can do at any time. Proper and complete milking requires proper preparation of the cows, a quiet, undisturbed atmosphere, milking at the same hours, and milking immediately after washing, wiping and massaging the udder, so that the effect of oxytocin is manifested throughout milking (Stelwagen, 2000).

The terms "frequency of milkings" and "interval between milkings" are often used. Reference frame time is 24 hours. When the number of milkings increases, the interval between milkings decreases and vice versa. Research has shown that the interval between two milkings must be less than 18 hours in order to avoid adverse effects on the product and quality of milk (Stelwagen et al., 1996, 1997). Milking twice a day has been a long practice in industrialized countries. In some countries milking passed to three or four times a day.

There are two physiological explanations for the impact of milking frequency on milk production (Stelwagen, 2001). The first explanation is the physical effect of the increase of intramammary pressure, which reduces the rate of milk synthesis in mammary epithelial cells.

The physical forces caused by the accumulation of milk in the mammary alveolus produce a compression of the secretory cells and thus reduces the metabolism of the cells and the synthesis of milk components. The rate of milk synthesis is the fastest immediately after milking, then with time it is reduced, and after 36 hours (if milking is not done) the milk synthesis stops (Cola & Cola, 2019). These phenomena, created by the increase of the intramammary pressure is avoided by increasing the number of milkings.

It has recently been observed that a hormone-like factor secreted by epithelial cells is involved in inhibiting milk synthesis. It has been called FIL; feedback inhibitor of lactation. As milk accumulates in the mammary gland between milkings, FIL inhibits the synthesis of milk components. By increasing the frequency of milking, this factor is removed from the mammary alveoli.

The general effect of milking frequency is also on secretion of prolactin. Circulating prolactin levels are highest immediately after each milking.

Research undertaken (Dahl et al., 2001) demonstrated that an increase of concentration of prolactin at the beginning of lactation can stimulate numerical growth of secretory cells in the mammary gland. Since milk production is a function of the number of secretory cells, starting milking with a larger number of secretory cells by default will increase production of milk. The important thing is that this increase will persist as cell loss is constant throughout the lactation. Prolactin has a stimulating effect on the development of breast cells and its higher concentrations large as response to increase of frequency of milking at the beginning of lactation explains the persistent effect of this practice on milk production.

The impact of milking 4x per day on milk components was studied by (Wiking et al., 2006), who found an increase in milk content in free fatty acids and fat globules with a larger diameter compared to milking 2x per day. Increasing the frequency of milking also causes a reduction in the number of somatic cells in milk (Dahl, 2004).

In the USA (Richard et al., 1994) were reported differences between milking three times a day (3x) and milking twice a day (2x) of 3.3 kg milk at primiparous cows and 3.5 kg at multiparous cows. Wall et al. (2007), in experiments on 1/2 udder with four milkings as compared to two find differences of 2.5 kg of milk at the beginning of lactation, the number of somatic cells, the percentage of fat and the percentage of protein being the same.

Van Baale et al. (2005) found no difference between milking six times a day and three

times a day. In an experiment with four milkings a day, they reported that after seven days the enzymatic activity of the secretory cells was not affected, but milk production was 18% higher than milking twice a day (Norgaard et al., 2005).
In a study using robotic milking in which cows have the freedom to be allowed to milk voluntarily, the average number of milkings was 3.9 times a day. It is considered that there is no biological advantage of increasing the frequency of milking more than four times a day (Ipema et al., 1987, quoted by Stelwagen, 2001).

MATERIALS AND METHODS

The aim of this research was to evaluate the effects of milking three times a day compared to milking twice a day in the first 28 days of lactation on total milk production.

Animals and feed rations

The experiment was carried out at S.C. FENOV SRL Dolj and included a total of 8 primiparous cows of Holstein Friesian breed (Table 1).

Table 1. Grouping of animals

Lactation	Daily milking frequency	No. of animals	Milking hours	Milking interval (hours)
1st Lactation	Group with 2 milkings	8	05:00 and 17:00	12
	Group with 3 milkings	8	06:00, 15:00 and 23:00	9 - 8 1/2 - 6 1/2
Total	Animals with 2 milkings	8	05:00 and 17:00	12
	Animals with 3 milkings	8	06:00, 15:00 and 23:00	9 - 8 1/2 - 6 1/2

The animals were kept "tied up" for 28 days, after which they were kept "free" in separate stalls. The feed ration was formulated according to NRC 2001 (Table 2), for cows of 600 kg live weight and production of 30 kg of milk per day with 3.60% fat and 3.35% protein. All feed ingredients were mixed once a day forming a total mixture ration and administered at discretion (Cola M., 2020), with a percentage of unconsumed residue of 5-7%.
Both primiparous and multiparous animals were randomly assigned to the two-milking and three-milking groups immediately after calving.

Registration of milk production and sample analysis

The animals were milked at the milking parlor, and milk production was recorded daily at each milking starting with the 3rd day of milking until the 7th day postpartum, after which, weekly, until the 28th day of lactation and every 13 weeks, until the end of lactation. After 28 days of lactation, from three milkings a day it was returned to two milkings a day, until the end of lactation.

Table 2. Ration ingredients and chemical analysis

Ingredients	Feed ration
Concentrate mixture	47,00
Corn silo	24,80
Alfalfa hay	20,20
Brewers grains	4,00
Soya beans	2,00
Vitamin-mineral premix	2,00
Chemical composition:	
Crude protein	17,00
Neutral detergent fiber	36,20

Milk samples were collected from each animal and each milking. Cows with decreased milk production on the control day (animals in rut), at one or more milkings, were excluded from the evaluations and introduced in the following weeks. The milk samples were kept in the refrigerator and analyzed within a maximum of 24 hours. On the day of the analyzes, the milk samples from each milking, both from cows milked twice a day and from those milked three times a day, were mixed in equal proportions, with only one sample per day. The fat and protein content of milk was determined in the animal husbandry laboratory of the Faculty of Agronomy in Craiova with the Ecomilk ultrasonic Milk Analyzers.

Statistical analyses

Milk production and fat and protein content were statistically analyzed. Statistical differences were recorded when the value $p \leq 0.05$.

RESULTS AND DISCUSSIONS

a) Milk production of primiparous animals

Table 3 shows the milk production of primiparous animals made after 28 days of experimentation. The average milk production at the end of the first 28 days of experiments

was 19.85 kg for primiparous milked twice times a day and 22.80 kg for those milked three times a day (Table 3). The difference between the two productions is 2.95 kg, a distinctly significant difference. The average milk production of primiparous milked three times a day was after 28 days 14.8% higher than the production of primiparous milked twice a day. The evolution of average productions after 7, 14, 21 and 28 days postpartum is shown in Figure 1.

Table 3. Milk production of primiparous cows

Milking frequency		Average milk production after:			
		7 days p.p. * (kg milk with 3.5% fat)	14 days p.p. (kg milk with 3.5% fat)	21 days p.p. (kg milk with 3.5% fat)	28 days p.p. (kg milk with 3.5% fat)
2x (two milkings)		17.10	18.50	19.15	19.85
3x (three milkings)		20.00	21.30	20.50	22.80
Difference : 2X-3X	kg	2.90	2.80	1.35	2.95
	%	+16.9	15.1	7.0	14.8
Statistical significance of differences		Significantly distinct p<0.01	Significantly distinct p<0.01	Insignificant p> 0.05	Significantly distinct p<0.01

*p.p. = postpartum

After the first 7 days, primiparous cows milked three times a day achieved an average of 20.00 kg of milk corrected to 3.5% fat, while those milked twice a day achieved an average production of 17.10 kg milk corrected to 3.5% fat. There is an increase of 2.9 kg of milk in primiparous cows milked three times a day (an increase of 16.9%). Statistically analyzed, this increase is distinctly significant (P<0.01).

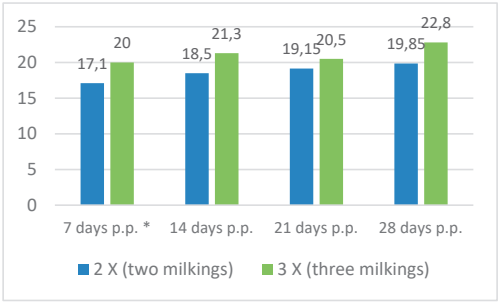

Figure 1. Milk production of primiparous cows

After 14 days, the primiparous cows milked three times a day achieved an average of 21.3 kg of milk corrected to 3.5% fat, and those milked twice a day produced an average of 18.50 kg of milk corrected to 3.5% fat while maintaining an increase of 2.8 kg of milk (15.1%) between the two groups.

After 21 days of experimentation, the average milk production of primiparous cows milked twice a day reaches 19.15 kg of milk with 3.5% fat, and of those milked three times a day at 20.50 kg of milk with 3,5% fat, with a decrease in production compared to the previous period by 3.8%, probably due to changes in genetic status.

After 28 days of experimentation, the average production of primiparous cows milked three times a day reached 22.80 kg of milk with 3.55% fat, and of those milked twice a day, 18.85 kg of milk with 3,5% fat, with a difference of 2.95 kg of milk (14.8%).

Statistically analyzed, this difference is distinctly significant (P<0.01).

After 119 days of lactation, the difference between the average milk production of primiparous cows milked three times a day (between 3 and 28 days of lactation) and the milk production of primiparous cows milked twice a day was 2.00 kg of milk with 3.5% fat (9.5%) (Figures 2 and 3).

Table 4. Average milk production of primiparous cows after 119, 210 and 301 days of lactation

Milking frequency		Average milk production after:			Milk production per 301 days of lactation	
		119 days p.p. * (kg milk with 3.5% fat)	210 days p.p. (kg milk with 3.5% fat)	301 days p.p. (kg milk with 3.5% fat)	Physically Kg	Maturity equivalent Kg
Two milkings a day (2X)		22.10	20.50	19.50	5869	7336
Three milkings a day (3X)		24.10	22.50	21.30	6411	8014
Differences 2X-3X	kg ±	+1.90	+1.80	542	678	678
	%	9.5	9.2	9.2	9.2	9.2
Statistical significance		Significant p <0.05	Significant p <0.05	Significant p <0.05	Significant P <0.05	Significant p <0.05

At 210 days, there was a difference of 1.9 kg of milk with 3.5% fat (9.2%), and at 301 days of lactation, the difference in production between the two primiparous groups was 1.8 kg of milk

with 3.5% fat (an increase of 9.2% in favour of primiparous cows milked three times a day during 3-28 days of lactation - Table 4).

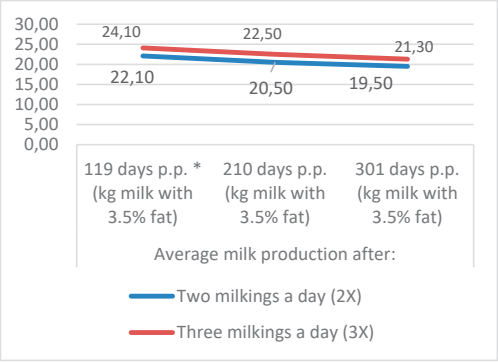

Figure 2. Average milk production of primiparous cows after 119, 210 and 301 days of lactation

Physical milk production per 301 days of lactation was 542 kg higher at 3 milks per day compared to 2 milks per days which means 9.2% (Figure 3)

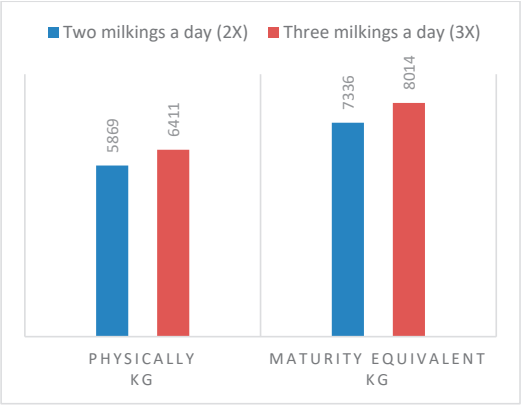

Figure 3. Milk production per 301 days of lactation

CONCLUSIONS

Milking the animals three times a day had the effect, in this experiment, of an increase in milk production both in the first 28 days of lactation and during 301 days of lactation.

The mammary gland of dairy cows has the ability to respond positively to the demands of milking three times a day.

Increasing milk production per cow increases the efficiency of milk production.

The milk production of a cow is determined by the number of secretory cells of the mammary gland and their metabolic activity.

The cows' response to milking 3 times a day takes place in stages, each stage reflecting different mechanisms.

In stage I, the increase in milk production occurs immediately due to the more frequent removal of the lactation inhibitory factor.

In stage II, the increase in milk production takes place in the short term (from a few days to one week), due to the stimulation of secretory cell differentiation.

In stage III, the increase of milk production takes place on a long-term basis (from a few weeks to a few months) due to the stimulation of the proliferation of secretory cells.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

STUDIES OF CAPONS MEAT PRODUCTION BELONGING TO THE HUBBARD CHICKEN HYBRID

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Abstract

The research aimed to evaluate the influence exerted by the removal of the testicles (orchidectomy) in roosters, on the meat production made by them. In this regard, were formed two batches of roosters belonging to the Hubbard chicken hybrid, one batch was experimental (LE), consisting of castrated roosters at the age of 6 weeks, and one control batch (LM) consisting of uncastrated roosters. The birds of the two groups were raised under identical conditions and received the same type of compound feed; their slaughter was performed at the age of 20 weeks, on which occasion the yield at slaughter, the share of anatomical parts in the composition of the carcasses, as well as the weight of the internal organs were established. The data obtained showed that castrated males had a higher slaughter yield compare to specimens uncastrated, with 0.68% higher for dressed yield on fresh carcass and with 1.01% higher for dressed yield on maturated carcass. Also, for castrated roosters, were registered higher values for participation rates for the anatomical parts with commercial interest (by 2.92% for the breast and by 6.15% for the thighs and drumstick); also, were registered higher weights for the internal organs (by 0.72% for heart, 29.04% for liver and 20.44% for gizzard). The conclusion of the study was that the application of caponisation to Hubbard roosters led to a higher yield at slaughter and higher participation rates for the anatomical portions with commercial interest (breast, thighs and wings) compared to uncastrated specimens.

Key words: *capon, dressed yield, Hubbard, internal organs, quantitative meat production.*

INTRODUCTION

Raising castrated roosters is more practiced in Italy, France, Taiwan, China and the United States, where they are marketed as high quality products (Sinanoglou et al., 2011). According to Regulation of European Commission no. 543/2008 of 16 June 2008, "a capon is a male bird that is surgically castrated before reaching maturity and slaughtered at a minimum age of 140 days".

Castration can cause changes in the aspect and comportment of roosters, but also changes in metabolism (Chen et al., 2006). Compared to uncastrated roosters, capons have a better metabolism and higher body weights, with 10-20%; their meat is tender and juicy (Sirri et al., 2009).

Castrated roosters are characterized by high deposits of adipose tissue, especially in the abdominal area, which is considered to have a beneficial effect on improving taste properties, an important aspect for todays consumers who increasingly demand products with remarkable organoleptic properties (Mast et al., 1981; Shao et al., 2009; Sinanoglou et al., 2011). At the same time, certain classes of consumers want extra quality products that stand out from conventional poultry products.

MATERIALS AND METHODS

The biological material was represented by 30 roosters belonging to the commercial chicken hybrid "Hubbard", divided into two batches: the experimental group (LE) composed of 20 heads. and the control group (LM) composed of 10 heads.

The difference among the two batches was represented by the fact that the roosters from the experimental group (LE) were castrated at the age of 6 weeks.

Castration of roosters was performed by the method of bilateral laparotomy in the last intercostal space, puncturing the air sacs, bring to the fore the testicles, by means of a special forceps, then performing orchidectomy by unlimited torsion (Figure 1). The wound suture was made in a continuous thread.

The roosters were raised in a space with a controlled environment, on permanent bedding; growth spaces were arranged that provided an area of 0.45 m^2/head, a drip water management device (1/head) and truncated feeders (20 cm/head). Both batches were fed at discretion, with the same type of feed, characterized by a protein content of 17% and an energy value of 2800 kcal/kg.

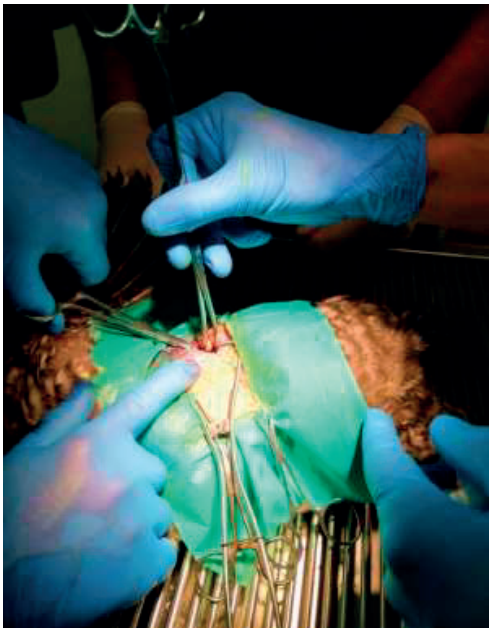

Figure 1. Torsion of the testicle

The birds of the two batches were slaughtered at the age of 20 weeks; which time the following parameters were determined:

• ***Yield at slaughter.*** It was calculated as the percentage ratio between the weight of live birds and the weight of carcasses resulting from their slaughter. It was calculated immediately after slaughter of the birds and 24 hours after slaughter, during which time the carcases were stored at a temperature of +2°C;

• ***The percentage of the trance portions participation (wings, brest, upper thighs, drumsticks, back with head and feets).*** Each trance portion was weighed individually, then reported to the weight of the carcass from which it came;

•***Weight of edible organs (heart, liver and gizzard).*** These were weighed individually, using the analytical balance.

The data obtained were statistically processed, calculating the arithmetic mean, the standard deviation of the mean and the coefficient of variation.

RESULTS AND DISCUSSIONS

Yield at slaughter

Before to slaughter, the birds were weighed and the values was noted in a document. Immediately after the suppression of life, the roosters were plucked and eviscerated. After performing these operations, the carcasses were weighed, then the yield at slaughter was calculated according to the formula.

For the control batch, was calculated an average value of 72.21 ± 2.24%, with a minimum value of 71.10% and a maximum value of 75.86%. The coefficient of variation registered a value of 3.10%, imprinting a homogeneous character for the batch (Table 2, Figure 3).

In the same mode was proceeded for the experimental batch (LE), its average value was 72.89 ± 4.55%; the minimum value calculated was 64.67%, and the maximum was 81.03%. The coefficient of variation was 6.25% (Table 1, Figure 3).

The carcasses were refrigerated for 24 hours at a temperature of +2°C; then they were weighed, and with the values obtained was calculated the yield at slaughter after refrigeration for both batches of roosters.

The control batch, after refrigeration, recorded an average value of the yield at slaughter of 70.78 ± 1.02%, with a minimum of 68.32% and a maximum value of 75.34%, the coefficient of variation being 3.81 %; while the experimental batch (LE) obtained an average of yield at slaughter after refrigeration of 71.79 ± 4.41%; the minimum value calculated was 63.90%, and the maximum value was 78.46%. The coefficient of variation was calculated to be 6.17%.

The percentage of the trance portions participation

After slaughter, the carcasses were refrigerated for 24 hours, then weighed and cut into anatomical portions (wings, breast, upper thighs, drumsticks, back with head and feets). In order to calculate the participation rate of

each anatomical portion, they were weighed and reported to the carcass weight (Figure 2).

Figure 2. Anatomical portion of two batches (left-LE, right-LM)

The experimental batch (LE) recorded for wings an average of 9.43% of the carcass weight, the breast represented from the carcass a percentage of 34.19%, the upper thighs represented 18.19% of the carcass weight, in time which drumstick recorded a percentage of 15.61%, and the back with head and feets accounted for 22.58% of the entire carcass (Table 3, Figure 4).

Regarding the control batch (LM) the proportions of the anatomical parts were: 9.26% wings, 32.97% breast, 16.97% upper thighs, 14.75% drumstick, 26.05% back with head and feets (Table 3, Figure 4).

Weight of edible organs

Immediately after evisceration, the edible organs (heart, liver, gizzard) were selected and weighed using the analytical balance.

In case of the experimental batch (LE), was recorded an average heart weight of 20.69 g, the liver weight was 75.92 g, and the gizzard weighed registered an average of 58.03 g (Table 4, Figure 5).

For the control batch (LM) the average heart weight was 20.54 g, the liver recorded an average weight of 53.87 g, while the gizzard weighed was calculated an average of 46.17 g (Table 4, Figure 5).

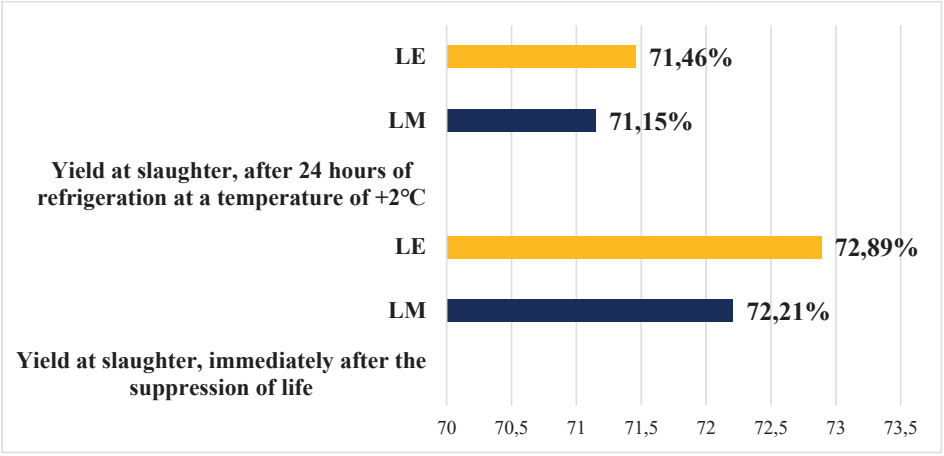

Figure 3. Average of slaughter yields obtained for the commercial hybrid "Hubbard"

Table 1. Yield at slaughter, immediately after the suppression of life

Batch	$\bar{X} + S_{\bar{X}}$ (%)	V %	Min. (%)	Max. (%)
Control	72.21 ± 2.24	3.10	71.10	75.86
Experimental	72.89 ± 4.55	6.25	64.67	81.03

Table 2. Yield at slaughter, after 24 hours of refrigeration at a temperature of +2°C

Batch	$\bar{X} + S_{\bar{X}}$ (%)	V %	Min. (%)	Max. (%)
Control	70.78 ± 1.02	3.81	68.32	75.34
Experimental	71.79 ± 4.41	6.17	63.90	78.46

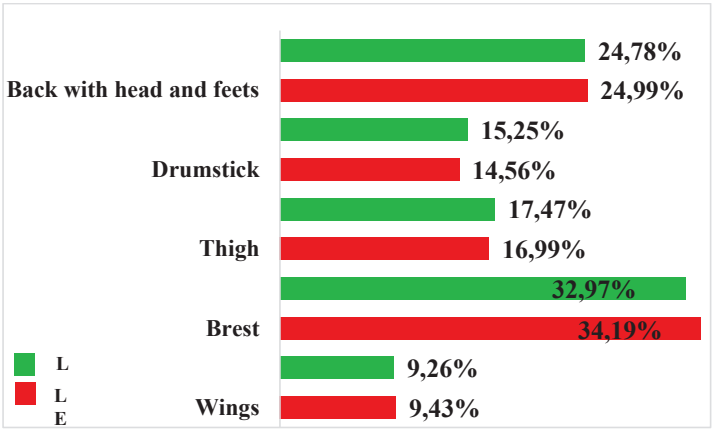

Figure 4. The percentage of portions cut into carcasses

Table 3. The percentage of the trance portions participation

Batch	Trance portion	$\bar{X} + S_{\bar{X}}$ (%)	V%	Min. (%)	Max. (%)
Experimental	Wings	9.43 ± 0.21	6.43	9.17	10.67
	Brest	34.19 ± 0.71	5.88	30.27	35.93
	Thigh	18.19 ± 0.28	4.70	15.63	17.50
	Drumstick	15.61 ± 0.14	2.64	13.15	15.26
	Back with head and feets	22.58 ± 0.91	10.35	22.93	26.15
Control	Wings	9.26 ± 0.10	2.86	9.07	9.79
	Brest	32.97 ± 1.36	10.95	27.97	35.36
	Thigh	16.97 ± 0.27	3.97	17.42	19.18
	Drumstick	14.75 ± 0.46	8.03	14.48	17.34
	Back with head and feets	26.05 ± 0.71	8.76	23.50	27.59

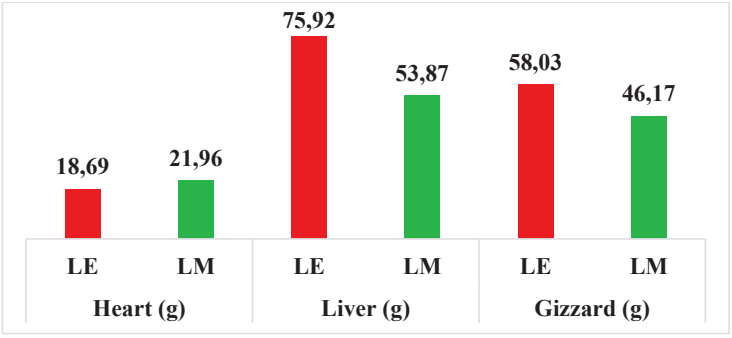

Figure 5. Average for weight of edible organs

Table 4. Weight of edible organs

Batch	Organ	$\bar{X} + S_{\bar{X}}$ (g)	V%	Min. (g)	Max. (g)
Experimental	Heart	20.69 ± 1.27	19.27	15.85	24.44
	Liver	75.92 ± 6.21	23.16	54.95	99.69
	Gizzard	58.03 ± 2.37	11.56	50.20	70.42
Control	Heart	20.54 ± 0.93	11.23	19.04	24.82
	Liver	53.87 ± 3.93	19.32	36.96	70.61
	Gizzard	46.17 ± 1.93	11.05	39.56	52.61

CONCLUSIONS

The results obtained after the slaughter of the capons obtained from the commercial chicken hybrid Hubbard led to the succeeding conclusions:

• regarding the yield at slaughter of fresh meat, for the experimental batch (LE) was calculated a higher value compared to the control batch (LM) by 0.68%;

• the value of the yield at slaughter after refrigeration, the experimental batch (LE) registered a value of 1.01% higher than the control batch (LM);

• the percentage of the trance portions participation the experimental batch (LE) registred for wings a value by 0.17% higher than the control batch (LM); regarding the share of breast participation in the carcass, the experimental batch (LE) registered an average value higher than the control batch (LM), with 1.22%. For the upper thighs the situation was identical to that of the chest. The proportion of the drumstick of the experimental batch (LE) was 0.86 higher than that of the control batch (LM). Regarding the share of participation in the carcass of back with head and feets, the situation was different, the control batch (LM) registering an average value higher by 3.47% compared to the experimental batch (LE);

• after weighing the fresh edible organs, it was observed that the experimental batch (LE) registered higher values, compared to the control batch (LM), for all three organs taken into analysis (heart, liver and gizzard); respectively 0.15 g for the heart, 22.05 g for the liver and 11.86 g for the gizzard.

The final conclusion of our study was that the application of orchidectomy (caponisation) to Hubbard roosters led to a higher yield at slaughter and higher participation rates for the anatomical portions of interest (breast, thighs and drumsticks) compared to uncastrated roosters.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

BREEDING AND PRODUCTION PARAMETERS OBTAINED FROM THE COMMON DUCK

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Abstract

Observations has been performed on biological material inside teaching waterfowl farm of the University of Agricultural Sciences and Veterinary Medicine Bucharest during December 2019 - June 2020 and results are compared with those recorded in year 2001 for groups of 60 birds of races Peking, Campbell khaki and Indian Runner. During this period there were monitored and analyzed the following breeding and production parameters: body weight, egg production, egg weight, fertility, hatchability, egg weight and day-old body weight. Peking Duck had an average body weight of 2358 g for females and 2696 g for males and an egg production of 120.42 eggs/bird and an egg weight of 77,62 g and a fertility of 87.34 % and a hatchability of 54.52% and a day-old body weight of 44.04 g. Campbell khaki Duck had an average body weight of 1683 g for females and 1954 g for males and an egg production of 189.77 eggs/bird and an egg weight of 68.37 g (significantly lower compared to 2001) and a fertility of 89.61% and a hatchability of 58.42 (significantly higher) and a day-old body weight of 35.05 g (significantly lower). Indian Runner Duck had an average body weight of 1791 g for females and 2040 g for males and an egg production of 173.74 eggs/bird and an egg weight of 69.86 g (significantly higher) and a fertility of 83.31% and a hatchability of 51.11% and a day-old body weight of 37.25 g (significantly higher).

Key words*: body weight, common duck, hatchability, hatching.*

INTRODUCTION

If rational raised waterfowls are bringing big and quick benefits and are able to use less costly feeds and are more resistant to diseases and are more quickly to respond to specific treatments (Cherry & Morris, 2008).

England has been world's first producer and breeder of Muscovy ducks. A type of Muscovy duck lighter than the Asian one has been created in England by crossings with races Aylesbury, Campbell, White Indian Runner etc. Cherry Valley from England is considered the biggest duck producing company in the world (Watt Poultry Statistical Yearbook, 2019; Creswell, 2002, 2013; Gerzilov et al., 2013).

Duck production is also highly developed in Netherlands which are exporting breeding material and duck meat in several European countries such as Germany and France.

In the Independent States Community where waterfowl production has a firm established tradition Peking duck is being produces from 1925. Populations of this race originating from different imports from England, China and Germany are being raised in several zones of this area such as Moscow, Kazakhstan, Byelorussia, Ukraine, etc. Generally adult ducks' weight is of 3-3.7 kg for females and 3.3-4 kg for males. Weight of young ducks rose for meat production has slightly large variations depending on population (Kinh et al., 2013).

Presently Peking is highly used for meat production both as pure race and as hybrids with other races. It has the disadvantage to produce too fat carcasses. Meat is very tender and juicy and it is well known worldwide for its pleasant taste.

Campbell race was created at the end of XIX century and it received the name of farm which created it and introduced it in English standard of poultry races in 1901 as a race with high egg production.

Campbell ducks were obtained from Indian Runner docks and local material and Mallard and Rouen ducks had also an important role in conception of this race. It was introduced in our country in 1949 and it was imported from Netherlands (Popescu-Micloşanu, 2004).
Campbell ducks are very precocious concerning both egg laying age and age of slaughtering for meat production. Eggs have good hatching results and they have high fertility and hatching capability (Linden, 2015).
It is one of the most widespread duck races in the world next after Peking because of their several qualities.
Indian Runner came from India where it came into being naturally and race was brought to England during last century. Color types are chestnut, chestnut with white spots, wild, black, chocolate, white, blue and trout colored (Van et al., 2000).
Hatching quality is good and fertility and hatchability are high.
This race is suitable for the improvement of egg production. It has been raised in parks or on different properties as a decorative bird for expositions or pure and simple for its beauty (Farrell D.J., 2000).

MATERIALS AND METHODS

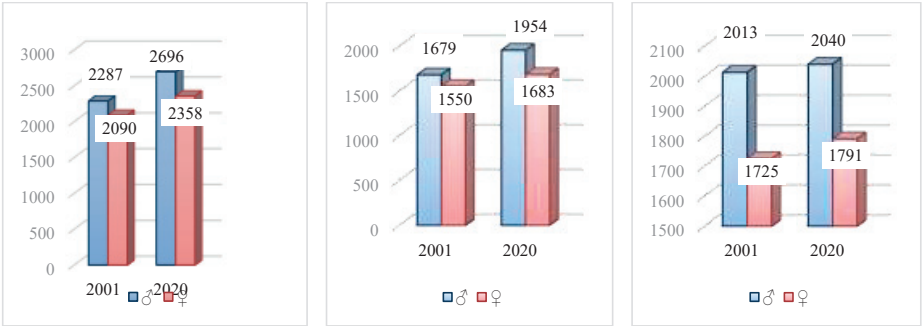
Researches were performed at Educational Farm Belciugatele - Waterfowl Farm which is located in Moara Domnească and it is belonging to University of Agronomic Sciences and Veterinary Medicine of Bucharest on three races of common duck respectively Peking, Campbell khaki and Indian Runner and 180 adult birds (60 bird/race) were studied in a proportion between sexes of 1:5 for which body weight and individual egg production were measured, s-au incubated eggs were weighted and main hatching parameters were evaluate. Some of the hatched ducklings have been kept to replace the parent flock and the others have been sold.
Study was carried out during an interval of six months (December 2019 and average yearly results were compared with those registered in year 2001 in the same Educational Farm of the University. These parameters (monitored weekly/monthly and on average by lying cycle) are as following: body weight (monthly - between December, 2019 - May, 2020) of adult birds, egg production/lying cycle (February - May), egg and chick weight (February - May), fertility and hatching (February - May).
Obtained data were statistically processed by classical means by calculating the average, variation, standard deviation, error of average and variability coefficient.
Student test has been used to study the significance of differences between calculated averages (between groups) (Sandu, 1995). Calculated Student test value has been compared with its critical (tabular) value at corresponding liberty degrees (cumulated liberty degrees n_1+n_2-2) and desired significance level ($\alpha = 0.05$; $\alpha = 0.01$; $\alpha = 0.001$; at a probability of 95%, 99% and respectively 99.99%).

RESULTS AND DISCUSSIONS

Production and breeding performances of the three studied races are presented in the followings.
In Peking duck:
- head is almost round with wide beak of orange color; torso has a rectangular shape and a characteristic oblique orientation; chest is rounded and uplifted; feathering is completely white;
- males average body weight is 2696 g and females average body weight is 2358 g with no significant differences between months and years; by fattening they can reach a body weigh of 3-4 kg;
- duckling's growth rate is very high;
- average egg production was 120.42 eggs by laying cycle and egg weight was 77.62 grams with no significant differences between months and years; eggs had white colored shells;
- fertility is very good - 87.34 % ranging between 82.84 - 90.35 with significant differences between months February - March and February - April;
- hatching percentage is 54.52%, ranging between 44.21 and 62.07% with clearly significant differences between months March - April and very significant differences between March - May;
- duckling's weight at day one age was 44.04 g with limits between 43.08-44.69 g and without significant differences.

Table 1. Body weight of common duck populations (grams)

Mention		Peking			Campbell khaki			Indian runner		
		2020	2001	Student 2001-2020	2020	2001	Student 2001-2020	2020	2001	Student 2001-2020
♂	X	2696	2287	2.0277	1954	1679	2.2446	2040	2013	0.1917
	sx	184.98	80.43		106.85	59.59		87.18	109.27	
	CV	15.35	7.86		12.23	7.93		9.56	12.14	
♀	X	2358	2090	1.4520	1683	1550	1.7896	1791	1725	0.3907
	sx	149.88	107.71		59.59	85.06		117.29	120.15	
	CV	14.21	11.52		7.93	11.30		14.64	15.57	



(a) (b) (c)

Figure 1. Body weight in common duck (a - Peking, b - Campbell khaki, c - Indian Runner, adults, g)

Table 2. Egg production in common duck (eggs/laying cycle)

Mention		Peking		Campbell khaki		Indian runner	
		Total	t 2001-2020	Total	t 2001-2020	Total	t 2001-2020
2020	X	120.42	0.7367	189.77	0.1571	173.74	0.5877
	sx	10.62		11.417		10.687	
	CV	17.64		12.03		12.30	
2001	X	110.14		192.38		182.64	
	sx	9.049		12.065		10.728	
	CV	16.43		12.54		11.75	

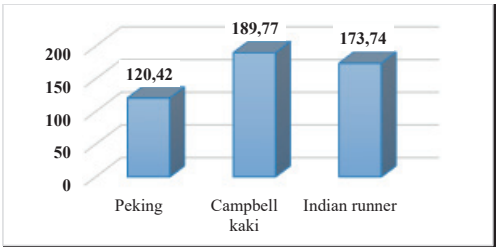


Figure 2. Egg production/lying cycle in common duck

The following morphological production and breeding characters have been analyzed in the population do Campbell khaki race:

- outside characters are external appearance of small race with torso having a cylindrical shape and a characteristic oblique orientation. Beak has a dark greenish color. Chest is rounded and uplifted and abdomen is bulky. Feet are being of average size and set apart at a good distance and feathering color is khaki;
- adult body weight is 1954 g for males and 1683 g for females with no significant differences between months and years;
- production characteristics: egg production is high of 189.77 eggs/laying cycle on average (with no significant differences) with eggs having an average weigh of 68.37 g with white and sometimes greenish shell (with significant differences between years);
- it is precocious race with egg laying starting at 5-6 months;
- fertility is very good - 89.61% with significant differences between months February - May and distinctive significant between February - March, February - April and between years;
- hatching percentage - 58.42%, ranging between 45.76 and 64.92%, with significant differences between years and with very significant differences between months March - April and March - May;
- ducklings weight at day one age - 35.05 g ranging between 34.24-35.57 g with significant differences between years.

Morphological, production and breeding traits of analyzed population of Indian Runner ducks were the followings:
- color types: brown with whit spots;
- adult ducks body weight is: female duck 1791 g and male duck 2040 g; with no significant differences between mounts and years;
- average egg production during last egg laying cycle: 173.74 eggs with an average egg weight of 69.86 g with significant differences between years;
- this race is very good for the improvement of egg production;
- it is starting to lay eggs at just 4 mounts of age;
- fertility is good - 83.31% with significant differences between months February - March, February - April;
- hatching percentage is 51.11% ranging between 40.31 and 51.11% with very significant differences between months March - April and March - May;
- ducklings weight at day one age - 37.25 g ranging between 36.38-37.81 g. with significant differences between years.

Table 3. Egg and ducklings' weight in common ducks (grams)

Mention		Peking			Campbell khaki			Indian runner		
		2020	2001	Student 2001-2020	2020	2001	Student 2001-2020	2020	2001	Student 2001-2020
Egg weight	X	**77.62**	**78.28**	**0.5614**	**68.37**	**73.34**	**3.6568***	**69.86**	**65.67**	**3.4141***
	s_X	0.799	0.869		1.036	0.881		1.018	0.688	
	CV	2.30	2.48		3.39	2.69		3.26	2.34	
Ducklings weight	X	**44.04**	**42.81**	**1.8712**	**35.05**	**38.29**	**4.6802***	**37.25**	**34.75**	**3.7696***
	s_X	0.454	0.475		0.541	0.430		0.544	0.378	
	CV	2.27	2.48		3.45	2.51		3.27	2.44	

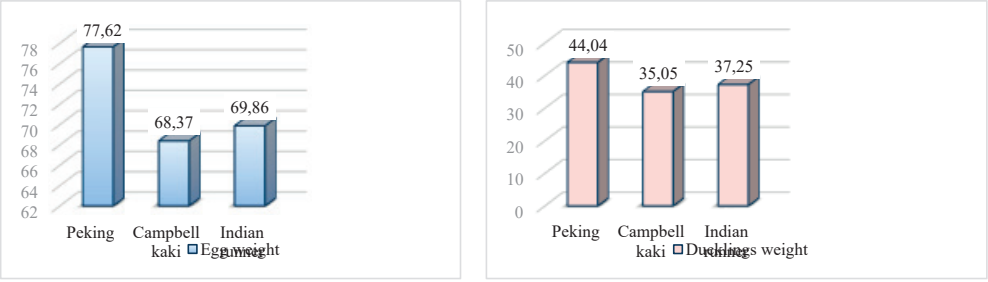


Figure 3. Average egg and ducklings' weight in common ducks (grams)

Table 4. Fertility (%) and hatchability (%) in common ducks

Mention		Peking			Campbell khaki			Indian runner		
		2020	2001	Student 2001-2020	2020	2001	Student 2001-2020	2020	2001	Student 2001-2020
Fertility	X	**87.34**	**84.16**	**2.1759**	**89.61**	**80.34**	**6.5215****	**83.31**	**88.28**	**2.5225**
	s_X	0.984	1.080		0.763	1.198		1.328	1.456	
	CV	2.52	2.87		1.91	3.33		3.57	3.69	
Hatching	X	**54.52**	**52.84**	**1.1267**	**58.42**	**51.38**	**5.0856***	**51.11**	**54.84**	**2.6492**
	s_X	1.259	0.80		1.184	0.717		0.970	1.02	
	CV	5.16	3.39		4.53	3.12		4.24	4.17	

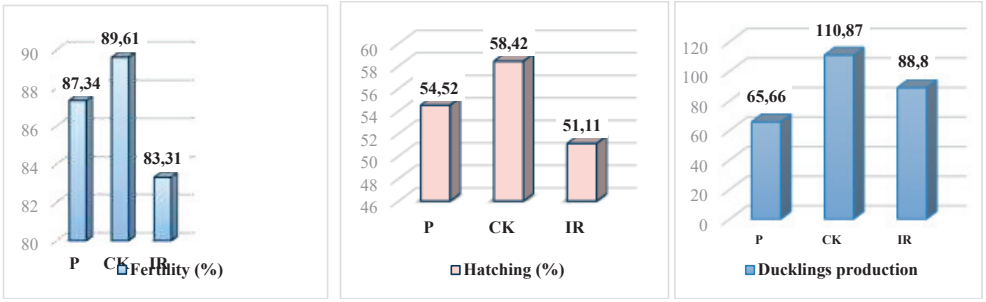


Figure 4. Fertility (%), hatching (%) and ducklings production by female in Muscovy duck
(P - Peking, CK - Campbell khaki, IR - Indian runner)

Comparative analyze of results revealed that males body weight ranged between 1954 g in Campbell duck and 2696 g in Peking duck; female body weight values had the same profile with 1683 g in Campbell ducks and 2358 g in Peking ducks.

Average egg weight ranges between 63.37 g in Campbell duck and 77.62 in Peking duck.

Fertility is high in all duck populations monitored and is ranging between 83.31 and 89.1. Hatching percentage had very good values ranging between 51.1-58.42% with highest value in Campbell ducks.

Ducklings' number by breeding female had average values ranging between 65.66-110.87 ducklings. In Peking Ducklings number by breeding female was 65.66 and biggest values have been obtained in small races: 88.80 ducklings by female were obtained in Indian Runner ducks and 110.87 ducklings by female were obtained in Campbell ducks.

CONCLUSIONS

Common duck races present in this study (Peking, Campbell khaki and Indian Runner) had production and breeding performances good and similar to those described in literature.

Flocks from the farm Moara Domnească are being a valuable gene pool which might be the foundation of both obtaining biological material suitable to be marketed as pure race (Peking and small races with good egg production) and producing mullards by crossing females of Peking lines with males of Muscovy duck lines.

ACKNOWLEDGEMENTS

This study was carried out with the support of the Ministry of Agriculture and Rural Development and it was financed inside the frame of project ADER 823/2019.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

BIBLIOGRAPHIC RESEARCH ON THE BIOLOGICAL VALUE OF MILK FROM DIFFERENT SPECIES OF DOMESTIC ANIMALS

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Abstract

The objective of the work is to carry out a bibliographic study on the biological value of milk from different animal species (cow, buffalo, goat, sheep, donkey). The quality of the milk and its composition varies depending on the breed, diet, feeding practices, management system, lactation stage and animal health. Milk and products obtained from it contain most of the nutrients needed by the human body, with a high biological value. Studies have shown that the milk with the highest fat content is sheep's milk, followed by buffalo milk, cow's milk, goat's milk and donkey's milk. In addition to the genetic variation between animal breeds, feeding fodder with a fat and fiber content can lead to an increase in the fat content of milk.

Key words*: biological value, chemical composition, milk, species.*

INTRODUCTION

Globally, consumers are paying attention to foods and their compositions due to a relationship between diet and human health (Rafiq et al., 2016). The history of milk and its products dates back to antiquity, from the time when man began to domesticate animals and raise them. 11,000 years ago, the first domestication of ruminants was attempted in the Middle East (Barłowska et al., 2011). Different regions around the world have adapted species to their area for milk production. Buffalo milk is often used in many parts of the world. The most recent nutritional finding refers to donkey's milk, which is similar to human milk in terms of protein composition (Barłowska et al., 2011). Milk is important in a healthy and balanced diet. It provides all the energy and nutrients needed to ensure proper growth and development (Pereira, 2014). The composition of milk is a necessary aspect that influences the quality of dairy products. The composition of milk varies significantly between different species. The quality of dairy products depends on the composition of the milk, which varies depending on the stage of lactation, milking methods, environment, season, diet, feeding system, breed and species (Kittivachra et al., 2007; Rafiq et al., 2016). Special nutritional characteristics have been identified for different types of milk. Underutilized resources are of great importance to dairy farmers, processors and consumers for designing innovative products with versatility, taste and functionality (Rafiq et al., 2016).

In Figure 1 milk production is dominated by 5 species of animals: cattle, sheep, goats, buffaloes and donkeys.

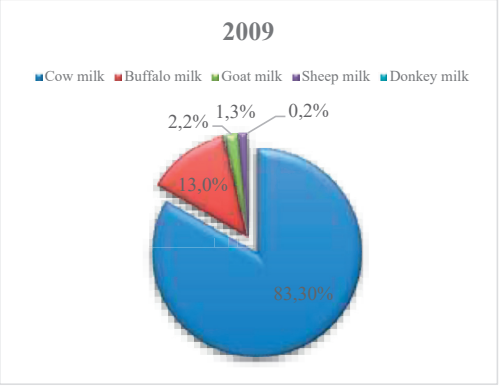

Figure 1. The proportion of milk globally in 2009, dominated by cattle, sheep, goats, buffalo, donkey. (Source: FAOSTAT, March 2021)

According to FAO statistical databases (2021) globally in 2009 of which 83.3% was in cow's

milk, 13% buffalo milk, 2.2% goat's milk, 1.3% sheep's milk and 0.2% milk of donkey.

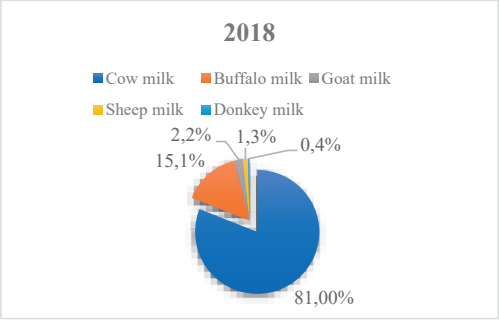

Figure 2. The proportion of milk globally in 2018, dominated by cattle, sheep, goats, buffalo, donkey. (Source: FAOSTAT, March 2021)

In 2018 it was 81.0% cow's milk, 15.1% buffalo's milk, 2.2 goat's milk, 1.3% sheep's milk and 0.4 donkey's milk. It is observed that buffalo milk increased and cow's milk production decreased slightly during this period (Figure 2).

MATERIALS AND METHODS

To achieve the objectives of this study, 40 bibliographic sources from the literature were consulted. Scientific databases relevant articles identified by keywords were consulted: chemical composition, milk, species, biological value and we selected based on the available literature articles that discuss comparative nutritional value analyzes of milk. The research methods used in this study were observed, analyzed and interpreted graphically data from the literature on the biological values of milk from different animal species. This analysis covered different animal species used for milk production purposes (cattle, sheep, goats, buffaloes and donkeys). The period analyzed in this study was 1970-2020. Scientific research has established that milk has a complex chemical composition, especially in terms of casein fractions, whey proteins, amino acids.

RESULTS AND DISCUSSIONS

Comparative milk compositions of cattle, sheep, goats, buffalo and donkey species

The data shown in Table 1 show a substantial variability of the basic chemical composition of milk from different animal species. An analysis of data from the literature was applied for the 5 species of domestic animals of the greatest importance in world milk production. The four major components of milk regardless of species are proteins, fats, minerals and lactose (Gantner et al., 2015) and to some extent minimizes the impact of factors that change the composition of milk, such as race, feeding system, stage of lactation or time of year, milking interval, type of food, and climate (Haenlein, 2004; Claeys et al., 2014). The energy value of milk is closely linked to the concentration of certain compounds in the dry matter, in particular the amount of fat (Barłowska et al., 2011).

Table 1. Basic chemical composition of milk from different animal species

Species	Protein %	Fat %	Lactose %	Ash %
Cattle[a]	3.87	4.96	5.00	0.80
Sheep[a]	5.50	6.80	5.22	0.98
Goats[a]	3.48	4.73	4.88	0.99
Buffalo[a]	4.78	8.41	6.21	0.98
Donkey[b]	1.72	0.38	6.88	-

Source: Adapted and modified from: [a]Mahmood & Usman, 2010; [b]Barłowska et al., 2011. The values found in the literature for different species of domestic animals (Arman et al., 1974; Watson et al., 2017)

Milk Proteins

In 2010, a comparative study of the physico-chemical parameters of milk from cows, buffaloes, goats and sheep was published by Mahmood & Usman. In this paper Mahmood & Usman tested the ash, fats, proteins, lactose in milk. It was found that all parameters tested were higher in buffalo and sheep's milk than in cow's and goat's milk. The protein content of buffalo's milk was higher than that of cow's and goat's milk but lower than that of sheep's milk at one level ($p<0.001$). There was a difference ($p>0.05$) between the protein content of cow's and goat's milk. The results of this paper in terms of protein content of cow's milk were similar to the results of Imran et al. (2008); Enb et al. (2009); Ahmed & Zubeir (2007); Abd Elrahman et al. (2009). The protein content found in sheep's milk was lower (5.47%) than that reported by Pavić et al. (2002). The reduction could be caused by the difference in race, the health of the udder and the stage of lactation. The protein content found in goat's milk was similar to that reported by

Strzałkowska et al. (2009). The protein content found in buffalo milk was similar to the results of Imran et al., (2008). A higher protein content in buffalo milk was reported by Braun & Preuss (2008) and Fundora et al. (2001). Barłowska et al. (2011) found that sheep's milk (5.73%) and buffalo's milk (4.48%) are the highest in terms of protein content, including casein and fat. Roy et al. (2020) found that sheep's milk (4.5-7.0 g 100 ml^{-1}) and goat's milk (3.0-5.2 g 100 ml^{-1}) have a higher protein content compared to buffalo milk (2.7-4.7 g 100 ml^{-1}) and cattle (3.0-3.9 g 100 ml^{-1}). Sawaya et al. (1984) studied the chemical composition and nutritional value of goat milk from two breeds, Masri and Aardi from Saudi Arabia. In both, Aardi and Masri milk components, including fat (2.83%, 3.06%) and protein (3.28%, 3.41%) were slightly lower than those of other species.

Kapadiya et al. (2016) found that sheep's and buffalo's milk has a higher content of protein and fat than other animal species. Goat's milk in protein content was statistically ($p<0.05$) than in cow's milk. In the fat content of goat's milk was ($p>0.05$) than that of cow's milk as well as buffalo's milk. Regarding the lactose content of goat's milk was ($p>0.05$) than that of cow's milk as well as buffalo's milk, donkey's milk contains the highest lactose content. Sheep's milk had the highest ash content, followed by goat's milk, and cow's milk had the lowest ash content. The fat content of goat's milk had an average value of 3.84%, in cow's milk it was 4.88%, of 8.30% in buffalo's milk. The fat content of goat's milk was ($p>0.05$) higher than that of cow's milk as well as buffalo's milk. The average value in lactose content was 4.16% in goat's milk, cow's milk was 4.76% and the average value was 4.86% in buffalo's milk. The lactose content of goat's milk was statistically ($p>0.05$) higher than that of cow's milk and buffalo's milk. The average value of the ash content was 0.89% in goat's milk. Similarly, the average value in cow's milk was 0.76% and 0.81% in buffalo's milk. Sheep's milk had the highest ash content and the lowest ash content was found in cow's milk. The average value in cow's milk is 0.76%. The average value in buffalo milk was 0.81%. (Kapadiya et al., 2016).

Proportions of major proteins

Milk proteins differ in composition and properties. They are divided into casein complexes and whey protein fractions. There are 4 main fractions of casein: αs 1 -, αs 2 -, β- and κ. Sheep's milk is the richest in the β-casein fraction 15.6-39.6 (g L^{-1}), followed by buffalo milk 12.6-20.9 (g L^{-1}). The smallest fraction of α s1-casein was recorded in goat laptops 0-13.0 (g L^{-1}) (Roy et al., 2020). Casein from cow's milk and buffalo milk is very abundant (38.4% and 30.2% of total casein, respectively, in the αs 1 fraction (Zicarelli, 2004). Goat's and sheep's milk has a lower casein-whey-protein ratio (82:18), as well as a relatively higher β-casein 0-29.6 (g L^{-1}); α s-casein 2.3-11.6 (g L^{-1}); compared to cow's milk (Roy et al., 2020). There are 2 types of major whey proteins β-Lactoglobulin and α-Lactalbumin. Sheep's milk was recorded with the highest concentration of β-Lactoglobulin 6.5-13.5 g L^{-1} and α-Lactalbumin 1-1.9 g L^{-1}, and its lowest concentration recorded in goat's milk 1.5-5.0 g L^{-1} β-Lactoglobulin and α-Lactalbumin 0.7-2.3 g L^{-1}. Milk casein micelles from different species differ in size, hydration and mineralization. Roy et al. (2020) reported that the size of casein micelles in goat's milk 180-301 nm, and sheep 180-210 nm, is larger than that in cow's milk 150-182 nm. It was considered that the hydration level of casein micelles was negatively correlated with the mineralization of micelles (Remeuf & Lenoir, 1986), i.e, when the mineralization of casein micelles increases, the degree of hydration of casein micelles decreases. The casein micelles in buffalo milk (Ahmad et al., 2008) and donkey milk are considered to be less hydrated and more mineralized than those in cow's milk. Thus, the differential between races, genetic variants and phosphorylation sites of caseins can be added to the variation of casein micelle characteristics and between species (Crowley, 2017).

Milk fat composition

Donkey's milk contains lower levels of saturated fatty acids, and proportions of polyunsaturated fatty acids than milk from other species. The highest cholesterol content was found in cow's milk between 13.1-31.4 mg/100 ml milk (Table 2).

One of the specific characteristics of ruminant milk is the presence of the CLA. The conjugated linoleic acid content and cholesterol content are higher in cow's milk than milk from other species. Sheep and goat milk fats are rich in short chain (responsibility for the distinctive flavor of these milks) and medium chain triacylglycerols (TAG); Similarly, buffalo fat contains higher proportions of medium-chain TAGs than cow's milk, which has higher proportions of long-chain TAGs (Ceballos et al., 2009; Ruiz-Sala et al., 1996; Jenness, 1980; Abd El-Salam, 2011).

Talpur et al. (2008) in their paper, the CLA content was subject to a variation ($p \leq 0.05$) depending on the milk of different species and season. The average CLA content was found to be between 0.40 and 1.10 g/100 g of total fatty acids, with a higher fat content of cow's milk between 0.45-1.10 g/100 g, followed of sheep 0.57-0.91 g/100 g, goat 0.41-0.64 g/100 g and fat from buffalo milk 0.39-0.63 g/100 g. Most of the CLA content contained the cis-9, trans-11 isomer which contributed to 84-92% of CLA, while trans-10, cis-12 accounted for 4-7% of CLA all animal species investigated in the study.

Donkey milk is characterized by a specific fatty acid profile. It contains several times more SFA (C8: 0, C10: 0 and C 12: 0), twice fewer fatty acids C 14: 0 and C16: 0. It contains very little stearic acid, C18: 0 (1.12%), while in the milk of other species it is about 12%. Oleic acid (C18: 1) deserves special attention among unsaturated fatty acids. In donkey milk the amount of oleic acid is three times less than in the milk of other species. As already mentioned, donkey milk is very rich in PUFA, C18: 2 and C18: 3, linoleic and linolenic (Barłowska et al., 2011).

Buffalo milk contains almost three times more C14: 0 myristic acid and twice as much C16: 0 palmitic acid as cow's, sheep's and goat's milk. A characteristic of goat's milk is a high concentration of short-chain fatty acids. Ceballos et al. (2009) reported that goat's milk fat compared to cow's milk fat contains 54.6% more C6:0, 69.9% C8:0, 80.2% C10:0 and 56.3% CLA and 75% less C4:0. The characteristic of sheep's milk is a higher concentration of butyric acid (C4:0) and conjugated linoleic acid (CLA) than cow's and goat's milk.

Table 2. Milk fatty acid profile of different animal species (% of total fatty acids) and cholesterol content

Fatty acids FA	Cattle	Sheep	Goats	Buffalo	Donkey
C 4:0[a]	3.54	4.06	2.46	3.90	0,60[b]
C 6:0[a]	2.21	2.78	2.40	2.33	1.22[b]
C 8:0[a]	2.32	3.13	2.53	2.41	12.80[b]
C 10:0[a]	3.52	4,97	11.07	2.40	18,65[b]
C 12:0[b]	3.83	3.35	4.45	3.09	10,67
C 14:0[a]	11.41	10.16	10.16	10.64	5,77[b]
C 16:0[a]	26,66	23.11	24.20	28.02	11.47[b]
C 17:0[a]	0.50	0.76	0.63	0.50	2.37[b]
C 18:0[a]	11.82	12,88	12.51	12.58	1.12[b]
Total ≤ C14:0[a]	31.37	28.42	31.37	24.76	
Total SFA (%)[c]	72,8	74,6	73,7	74	67,7
C 14:1 c9cis[a]	0.84	0.58	0.22	0.67	
C 16:1 c9 cis[a]	1.68	0.39	0.67	1.56	
C 18:1 c9 cis[a]	24.72	23.32	22.03	24.10	
Total MUFA (%)[c]	30,3	24.29	35,9	29.4	35.0
C 16:1 trans[a]	0.31	0.29	0.38	0.37	-
C 18:1 t11 trans[a]	2.01	2.69	1.69	2.00	-
C 18:2 t9, t12 trans[a]	0.45	0.44	0.50	0.49	-
Total TFAa	2.76	3.15	2.66	2.66	-
C 18:2 c9, t11 (CLA)[a]	0.59	0.60	0.43	0.39	-
C 18:2 t10, c12 (CLA)[a]	0.036	0.032	0.024	0.027	-
Total CLA (%)[b]	0,45	0,67	0,68	0,49	-
C 18:2 cis[a]	1.96	1.17	0.70	1.55	8.15[b]
C 18:3 n-3[a]	0.70	0.92	0.82	0.68	6,47[b]
C 20: 4 n-6[a]	0.21	0.20	0.32	0.35	0,07[b]
C 20:5 n-3[a]	0.15	0.09	0.11	0.18	0,27[b]
C 22:6 n-6[a]	0.08	0.08	0.09	0.12	0,30[b]
PUFA (%)[b]	3.20	2.45	4.08	2,67	16.60
Cholesterol (mg / 100 ml lapte)[c]	13.1-31.4	14 -29.0	10.7-18.1	4-18,0	-

Source: [a]Talpur et al. (2008); [b]Barłowska et al. (2011); [c]Roy et al. (2020).

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; CLA, conjugated linoleic acid, TFA, drawn fatty acids.

Cholesterol is present in the globular membrane of milk fats (MFGM) and accounts for 95% of sterols in milk fat (Parodi, 2004). Barłowska et al. (2011) recorded the lowest cholesterol content (6.5 mg/100 g milk), in buffalo milk and the highest cholesterol content was in cow's milk (25.60 mg/100 g milk).

The fat content was 7.70-7.74%, and the protein content was 4.19 to 4.37%. The calcium and phosphorus content is higher than in cow's milk. Palmitic acid and oleic acid are the most important fatty acids in buffalo milk. Compared to the fat in cow's milk, buffalo fat is higher in butyric acid, palmitic acid, stearic and polyunsaturated fatty acids and lower in medium chain fatty acids C6-C12 (Vidu et al., 2015).

Milk lipids

Lipids form inclusions, which gradually increase in size and eventually migrate to the top of the cell from where they are discharged as globules into the collecting lumen

(Barłowska et al., 2011). The fat in the milk of all species is present in the form of small spherical droplets, called globules. Milk fat globules have an average diameter of less than 0.1 μm to about 18 μm. The size of these fat globules varies between the milk of different species. Differences in the size of milk fat cells from different species can influence the digestion of their fat differently (Gantner et al., 2015; Claeys et al., 2014).

Table 3. Size of milk fat globules from different mammal species

Species	Cattle[a]	Sheep[a]	Goats[a]	Buffalo[a]	Donkey[b]
Fat globule diameter (μm)	3.95	3.78	3.19	8.70	1-10

Source: [a]Barłowska et al. (2011); [b]Roy et al. (2020)

Fat globules with the largest average diameter are found in buffalo milk (8.70 μm), the smallest in goat's milk (3.19 μm) (Table 3). Attaie & Richter (2000) observed that the mean globe of milk fat in goat's milk was 2.76 μm (ranging from 0.73 to 8.58 μm) and 3.51 μm for cow milk (with a range of 0.92 to 15.75 μm). The fat globules in goat's milk occupy an area of 21,778 cm^2/ml, while in holiday milk this area is 17,117 cm^2/ml. Approximately 90% of all goat's milk fat globules have a diameter of less than 5.21 μm, while 90% of cow's milk globules have a diameter of less than 6.42 μm (Barłowska et al., 2011).

Milk lactose

The lactose content obtained by Mahmood & Usman (2010) was in the range of 4.56-6.21% in buffalo milk, 4.01-5.00% in cow's milk, 3.70-4.88% in goat's milk and 4.37-5.22% in sheep's milk. The amount of lactose content in buffalo milk was higher than in cow's and goat's milk at one level ($p<0.001$). A difference ($p<0.01$) was obtained between the lactose content of buffalo milk and sheep's milk. Difference ($p>0.05$) between the amount of lactose content in cow's, goat's and sheep's milk. The lactose content found in buffalo milk was similar to that obtained by Imran et al. (2008) and Khan et al. (2007). The results of Samia et al. (2009) were similar to those of Mahmood & Usman (2010) and Lingathurai et al. (2009). The highest lactose content was obtained in buffalo milk (6.21%) followed by goat's milk (4.88%), the lowest being recorded in cow's milk (5.00%). The lactose content of goat's milk (4.88%) reported was similar to that reported by Imran et al. (2008), Strzałkowska et al. (2009), Bhosale et al. (2009) and Sawaya (1984). The lactose content reported by Mahmood & Usman (2010) in sheep's milk (5.22%) was similar to that reported by Pavić et al. (2002) and Bylund (1995).

Milk ash

The results of Mahmood and Usman (2010) in the ash content was in the range of 0.69-0.98% in buffalo milk, 0.40-0.80% in cow's milk, 0.56-0.99% in goat's milk and 0.78-0.98% in sheep's milk. Cow's milk in ash content was lower than in buffalo and sheep's milk at one level ($p<0.001$). The difference ($p<0.05$) was between the amount of ash in cow's and goat's milk. There was a difference ($p>0.05$) between the amount of ash in buffalo milk, goats and sheep. The amount of ash found in buffalo milk (0.98%) was in agreement with that reported by Enb et al. (2009), Khan et al. (2007), Imran et al. (2008) and Han et al. (2007). The amount of ash found in cow's milk (0.80%) was in line with that reported by Enb et al. (2009) and Imran et al. (2008). The amount of ash content found in goat's milk (0.98%) was consistent with the results of Bhosale et al. (2009) and Keskin et al. (2004). The ash content found in sheep's milk (0.98%) was similar to that reported by Adewumi & Olorunnisomo (2009) and Bylund (1995).

Milk mineral components

The main mineral compounds in milk are calcium and phosphorus. The high bioavailability of these minerals influences the unique nutrient of milk (Barłowska et al., 2011). The highest concentration of it and other minerals is specific to sheep's milk; whereas donkey's milk contains the smallest amounts of these compounds (Table 4). Goat's milk is characterized by the lowest concentration of iron, zinc and copper (Ljutovac et al., 2008).

Table 4. Selected mineral content of milk from different species

Species	Parameters									
	Calcium (Ca) (mg / 100 g)[a]	Phosphorus (P) (mg/100 g)[a]	Magnesium (Mg) (mg /100 g)	Potassium (K) (mg/100g)[a]	Sodium (Na) (µg/100 g)[a]	Zinc (Zn) (µg/100 g)[a]	Iron (Fe) (µg/100 g)[a]	Copper (Cu) (µg/100 g)[a]	Manganese (Mn) (µg / 100 g)	Iodine (I) (µg / 100 g)
Cattle	122	119	16.54[b]	152	58	530	80	60	20	2.1
Sheep	195-200	124-158	18–21[a]	136-140	44-58	520-747	72-122	40-68	5.3-9	10.4
Goats	132	97.7	21.16[b]	152	59.4	370	60	80	6.53	-
Buffalo	112	99	21.40[b]	92	35	410	161	35	-	-
Donkey	67.67	48.7	3.73[a]	49.72	21.83	-	-	-	-	-

Source: [a]Barłowska et al. (2011); [b]Kapadiya et al. (2016).

The mineral content selected from goat's, cow's and buffalo's milk by Kapadiya et al. (2016) was as follows: the calcium content determined in five replicates had an average value of 129.08 mg/100 ml in goat's milk. In cow's milk, the average was 120.24 mg/100 ml, in buffalo milk 178.59 mg/100 ml. The calcium content of buffalo milk was higher ($p>0.05$) than that of goat's milk and cow's milk. The magnesium content had an average of 19.94 mg/100 ml in goat's milk, in cow's milk an average value of 12.65 mg/100 ml and an average value of 18.29 mg/100 ml in milk of buffalo. The magnesium content of goat's milk was higher ($p>0.05$) than that of cow's milk. The magnesium content of goat's milk was statistically insignificant ($p<0.05$) with buffalo's milk. The phosphorus content had an average value of 98.91 mg/100 ml in goat laptops, in cow's milk, an average value of 88.08 mg/100 ml and an average value of 109.22 mg/100 ml in buffalo milk. The differences in phosphorus content from three types of milk studied by Kapadiya et al. (2016) were found to be statistically insignificant ($p<0.05$). Regarding the chloride content, Kapadiya et al. (2016) obtained an average value of 0.16% in goat's milk, in cow's milk an average value of 0.13% and an average value of 0.11% in buffalo milk.

Belewu & Aiyegbusi (2002) published a comparative article on the mineral content of milk from humans, cows and goats. It was found that the highest content of Ca, Mg, P, Fe, Cu and Mn was identified in goat's milk and human milk, and milk from cows had the lowest content. The mineral content in goat's milk was as follows: Na (210.41 mg/100 g), K (1.55 mg/100 g), Ca (5.56 mg/100 g), Mg (2.30 mg/100 g), P (1.20 mg/100 g), Zn (0.80 mg/100 g). The mineral content in cow's milk was as follows: Na (51.92 mg/100 g), K (1.30 mg/100 g), Ca (4.03 mg/100 g), Mg (1.03 mg/100 g), P (0.92 mg/100 g), Zn (0.11 mg/100 g).

In the paper by Vidu et al. (2015) it was found that the distribution of minerals depends on the variable number of lactations. Calcium is found in the largest proportion in buffalo milk obtained from the third lactation (1.21 $g.kg^{-1}$). Previous studies on different breeds have shown the following distribution of calcium levels: Murrah -0.83 $g.kg^{-1}$, Mediterranean breed 0.99 $g.kg^{-1}$, Jafarabadi -0.95 $g.kg^{-1}$ and half Murrah x Mediterranean -0.94 $g.kg^{-1}$. Differences were found between the mean values of phosphorus in buffalo milk in the first lactation (0.67 $g.kg^{-1}$), the 2nd lactation (0.76 $g.kg^{-1}$) and the 3rd lactation (0.59 $g.kg^{-1}$). Depending on the season, the level of distribution of phosphorus in the Mediterranean buffalo breed varied between 0.63 $g.kg^{-1}$ in winter and 1.10 $g.kg^{-1}$ in summer.

CONCLUSIONS

Although milk from all mammals contains the same main components, the composition may vary between different species, between different breeding variants within the same species and between individual animals. Milk is a source of food with a high biological value. The highest protein and fat content is found in sheep and buffalo milk. In terms of lactose content, donkey's milk contains the highest amount. Goat's milk had the highest ash content, followed by buffalo's milk. Regarding the content in polyunsaturated and monounsaturated fatty stocks, the highest content was recorded in sheep's milk. The conjugated linoleic acid content and cholesterol content is the highest in cow's milk. The main mineral compounds in milk are calcium and

phosphorus. The highest concentration of these and other minerals is specific to sheep's milk.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

PARASITES DISEASES DETERMINATION IN AN UNTREATED LOCAL POPULATION OF *APIS MELLIFERA* FOR IT'S NATURAL RESISTANCE DEVELOPMENT

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Abstract

Over the last few years, the honeybees faced a significant decline worldwide. Despite many biotic and abiotic stressors, one of the leading causes of honeybee colony loses is Varroa destructor, followed by Nosema spp.. Given the importance of honeybee in agriculture and a significantly increased resistance to treatments identified in Varroa destructor, a more sustainable method to counter this mite is needed. One of these sustainable methods is breeding for resistance to Varroa destructor. Due to the rising number of honeybee populations with potential resistance to Varroa destructor, a new promising breeding plan for natural selection was proposed. This breeding plan was adapted and implemented on a local population of honey bees in Transilvania, with the primary objective of obtaining resistant colonies to Varroa destructor. Development of these colonies was observed, and analysis of Varroa infestation level and Nosema spp. was performed to assess the health status of the population or the cause of mortality.

Key words*: honeybees, Nosema spp., Varroa destructor.*

INTRODUCTION

Given the importance of the honey bee in the ecosystem, the number of stressors becomes concerning (Goulson et al., 2015; Havard et al., 2020). For *Apis mellifera,* among the biotic stressors, there is present a wide range of natural pathogen agents as well as newly introduced pathogen agents such as *V. destructor* and *N. cerane* as an effect of the lack of many restrictive measures during the transport of biologic material over long distances (Oldroid, 1999; Paxton et al., 2007; Goulson et al., 2015).

For the biotic stressor, *V. destructor,* it was observed an increasing resistance to the treatments (Thomson et al., 2003; Pettis, 2004). Currently, there is no available treatment able to remove the parasite from the hive; thus, the mite is already selected for resistance to treatments (Pettis, 2004; Dieteman et al., 2012; Kamler et al., 2016). Furthermore, without additional ways to counter the mite, it will be only a matter of time until many of the remaining treatments will have reduced effectiveness (Dieteman et al., 2012). Over one active season, the reproductive cycle of *V. destructor* allows multiple generations of mites and allows the mite population to fixate alleles for resistance to acaricides inside one colony (Beaurepaire et al., 2017)

Moreover, if beekeepers count only on chemical treatments, in time, this will lead to an increase in the dosage and number of treatments despite alternating the treatment (Dieteman et al., 2012; Rinkevich, 2020).

In the case of *Nosema* spp. we have now present in Europe two pathogen agents, one represented by *N. apis* present on *A. mellifera* and the second one represented by *N. ceranae.* Given the difference in symptomatology, the exact time of arrival in Europe for *N. ceranae* in Europe is unknown (Paxton et al., 2007; Higes et al., 2010). Moreover, it is suggested a synergic effect between *Varroa* infested colonies and the level of infestation with *Nosema spp.* (van Dooremalen et al., 2018).

Among the alternative ways to fight against *V destructor* among the best long-term solutions is breeding for *V. destructor* resistance (Dieteman et al., 2012).

Among the reasons that favour the honey bees we have the *A. mellifera* genome witch according to literature, seems to have a high

recombination rate (Beye et al., 2006) and is suggested as an adaptative measure to increase the variation inside the colony in order to slow the spread of pathogen agents and increase colony performance and fitness (Gadau et al., 2000; Beye et al., 2006). Moreover, adding polyandry as the queen mates with 8 to 10 drones leads to a higher diversity between offspring (Fuchs & Moritz, 1999).

The emergence of the different populations across the globe that manage to resistant despite the lack of treatments presents another reason to support the idea of a breeding plan. Here we include VSH (Varroa Sensitive Hygiene) (Harbo & Harris, 1997) and Primorsky Russian honeybee (Rinderer et al., 2001; Rinderer et al., 2010) in the USA which were selected for resistance and the feral population of honey bees from Arnot forest (Seeley 2007; Seeley et al., 2015).

In Africa, it seems that populations of *A. mellifera capensis* and *A. mellifera scutellata* posed resistance to varroa infestation (Martin & Kryger, 2002). Moreover, it is suggested that *A. mellifera scutellata* seems to resist even more pathogen agents are present simultaneously (Strauss et al., 2013). And this trait seems to be passed on to the Africanized honey bees to. (Martin & Medina, 2004).

In Europe, we have Gotland population obtained from apiaries placed on Gotland Island and left untreated (Fries et al., 2006; Loke and Fries, 2011). Avignon's population was composed of bees that survived without treatment and untreated bees from different beekeepers (Le Conte et al., 2007; Le Conte et al., 2020). Toulouse population obtained from queens of *Apis mellifera intermissa* brought from Tunisia (Kefuss et al., 2004). Similarly, in the Østlandet region, Norway a population of untreated bees of mixed origin (Buckfast) was used to obtain bees that manage to survive despite lack of treatments (Oddie et al., 2017).

Over time breeding plans become available; in the case of Russian honey bee and Varroa Sensitive Hygiene, the programs reached a commercial level (Rinderer et al., 2010). In Europe, we have significant progress was made with the AGT program (The Arbeitsgemeinschaft Toleranzzucht) that began in 2003 and the BLUP (best linear unbiased prediction model that was adapted for *A. mellifera* specific reproductive particularities (Bienefeld et al., 2006; Büchler et al., 2010).

However, more recently, it was proposed a new breeding protocol. One that has as the main focus of obtaining resistant populations based on principles of natural selection and was adapted to the particularities of reproductive biology. Another important advantage is the equipment required for implementation, as in this case is represented by standard equipment that should be available in a standard apiary (Blacquière et al., 2019).

MATERIALS AND METHODS

Obtaining the population

Based on Blacquière et al. protocol, we managed to obtain 25 colonies with unrelated queens from different areas of the Transylvania region in the spring of 2019 (Blacquière et al., 2019). With these colonies, we tried to establish a new population of honey bees.

These colonies were left untreated, and we monitored their development.

From a total of 25 colonies, 14 colonies developed better and responded to the presence of the indicator frame. As a result, these colonies were selected, and we proceeded with the breeding plan. Each colony was split into 3 new colonies, and for each hive, we adjusted an equal portion of the population, brood and food resources.

Newly formed colonies were taken to a new location prepared in advance. The new apiary is located on the coordinates (46°44'15.31"N 23°37'10.45"E); the land surrounding this location belongs to the Research and Development Station for Fruit Growing within USAMV Cluj-Napoca. Since the new site was in a more isolated area and plenty of drones were available in each colony, we expect that most of the mating took place in close proximity of the hive (Moritz et al., 2007; Jaffé et al., 2010). We also added two natural swarms caught in Cluj-Napoca in this period.

The other 11 colonies were returned to the bio-base. To confirm the mating's success for a new colony was confirmed mated only after identifying the queen and the presence of eggs and fresh larva.

Over winter, we proceeded to make regular checks based on standard beekeeping practices.

From the new population of honey bees, we took samples at the beginning of February.

***Varroa* mite analysis**

In order to identify the level of infestation with *V. destructor*, we used the 75% ethanol wash method (Dietemann et al., 2013).

For each hive, we took around 300 bees and placed them in a jar. We added sufficient ethanol to cover the bees. Once the jar was closed, we shake for approximatively 90 seconds to dislodge the mites. The next step was to separate the mites and ethanol from the bees using two layers of mesh. The first layer had large gaps that allowed mites and alcohol to goo thought but separated the bees, while the second layer kept only the mites.

For better precision, after we counted the mites, we counted the total number of bees and checked the bee abdomen for mite presence.

The total number of mites was divided by the total number of bees to determine the exact proportion of infested individuals. This value was then multiplied by 100 to obtain the % of mite infestation /100 bees presented in Table 1.

***Nosema* spp. microscopic analysis**

Identification and analysis for *Nosema* took place at APHIS-DIA laboratory from USAMV Cluj-Napoca. Sample processing was made using the method recommended by the OIE manual (World Organisation for Animal Health, 2018). We used 60 worker abdomens/ hive. The abdomens were crushed using a mortar and a pestle using ultrapure 60 ml H_2O until we obtained a homogenous suspension. The suspension was then filtered through two layers of muslin and centrifuged for 6 minutes at 2700 rpm in order to remove debris. Pellets are resuspended in ultrapure H_2O to restore the initial dilution.

Sample analysis for *Nosema* spp. spores was made using a Bürker-Türk Counting Chamber and a microscope Nikon Eclipse 50i, Ob. 40x available at the laboratory.

The total number of spores/bee was using the standard formula:

$$Z = \alpha/\beta \times \delta \times 250{,}000$$

Z = represents the number of spores/bee;

α = is the total number of counted spores;

β = is the number of squares counted;

δ = represents the dilution factor;

250,000 = represents the volume for each counted square and is usually present on the counting chamber.

Table 1. Hives that reached the winter season and values of *Varroa* and *Nosema* infestation

Hive Code	Status	Sampled bees	*Varroa* infestatio/ 100 bees	Total number of spores/bee
H50	ok	yes	0.000%	11,000,000
H46	jan-feb	yes	4.762%	2,875,000
H59	feb-mar	yes	2.239%	52,750,000
H48	jan-feb	yes	2.913%	42,375,000
H49	ok	yes	0.699%	15,500,000
H43	feb-mar	yes	0.769%	46,625,000
H44	jan-feb	yes	3.000%	32,875,000
H63	jan-feb	yes	5.172%	3,125,000
H45	feb-mar	yes	3.175%	11,625,000
H47	jan-feb	yes	6.667%	4,000,000
H10	feb-mar	yes	0.730%	11,250,000
H65	jan-feb	yes	1.961%	49,500,000
H40	jan-feb	yes	5.660%	41,500,000
H42	jan-feb	yes	2.727%	29,625,000
H58	jan-feb	yes	0.901%	11,000,000
H24	jan-feb	yes	12.150%	18,875,000
H22	jan-feb	yes	5.882%	7,875,000
H26	feb-mar	yes	3.333%	10,875,000
H35	jan-feb	yes	1.563%	7,875,000
H38	feb-mar	yes	1.198%	64,500,000
H60	jan-feb	no	na	na
H25	jan-feb	yes	6.107%	2,875,000
H7	jan-feb	no	na	na
H39	ok	yes	2.667%	12,375,000
H32	**ok**	**yes**	1.000%	**42,250,000**
H67	ok	yes	0.000%	2,625,000
H34	ok	yes	2.500%	8,125,000
H53	**ok**	**yes**	0.000%	**101,375,000**
H37	jan-feb	yes	1.626%	2,000,000
H17	jan-feb	no	na	na
H20	feb-mar	yes	0.667%	15,375,000
H54	feb-mar	yes	1.754%	16,625,000
H62	jan-feb	no	0.000%	na
H57	jan-feb	no	3.030%	na
H55	ok	yes	0.000%	3,625,000
H56	feb-mar	yes	na	12,125,000

*na = no data aviable

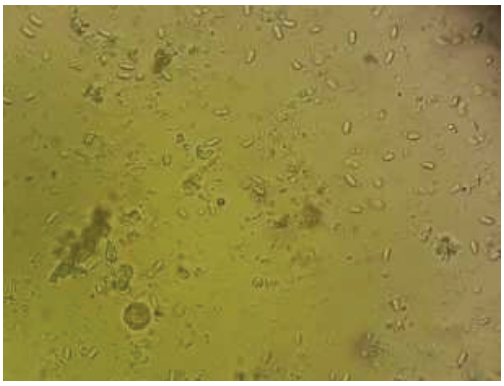


Figure 1. Preliminary test under the microscope before proceeding to the counting chamber step

All data was centralised and presented in Table 1. One colony was considered positive for *Nosema* spp. if the total number of spores exceeded 9 million spores/bees when the formula was applied (Chioveanu et al., 2009; Dumitru et al., 2020).

RESULTS AND DISCUSSIONS

Since the split was done on the natural mating season, we had available between 3 and 5 queen cells/new hive. Between 25-27 of May was the first check to confirm the queen presence and reproduction success in the interval. At this date, we confirmed the presence of brood and queen for 24 colonies. (marked with green in the supplementary table, queen presence and eggs column).

At the second inspection after one week, we confirmed mating success for the other 19 colonies (marked with purple in the supplementary table). The remaining two colonies were inspected at the third inspection one week later and confirmed the reproduction's success (marked with blue).

After the third inspection for queen presence, we confirmed a 100% success rate for queen mating with this protocol as all our colonies managed to have a newly mated queen.

Over summer, we monitored the development of these colonies, as presented in the summary table. We increased or decreased the number of frames based on each colony's available population, as shown in Supplementary Table 1.

At the end of the winter preparations, we ended up with 36 colonies. For the other 8 colonies, we noticed massive depopulation, and they had to be removed from the breeding program. (these colonies are marked as removed on the supplementary table). For the removed colonies, it can be noticed that colonies which developed well or manage to maintain over the active season suddenly collapsed in the population size and less than two frames with bees were available.

As a result, in winter, we entered with 36 colonies and the average size based on the mean between the number of frames from all hives was 5.25 frames/colony.

Over winter, we proceeded with external observations for the hives.

At the first major inspection for 2020, we confirmed the death of 19 colonies. For 6 of these colonies, we cannot confirm the brood's presence as presented in the supplementary table. However, for the rest, we could clearly identify the presence of brood in different stages.

At a second major inspection, we confirmed another mortality for one colony. They were followed by the other 4 at the end of February. In all cases, we had brood present. (Supplementary figure 1).

Despite being the first season without treatment, such high mortality levels lead us to analyse nosemosis as only varroosis infestation could not explain this mortality level.

Since both parasites reduce the worker's lifespan and contribute to some degree to immune suppression, the mortality rate for the population will be increased (Kurze et al., 2016; van Dooremalen, 2018), resulting in a decrease in the total population size.

This could explain the colony loss between January and February, with a small cluster and brood present on the frame. If the size of the bees' cluster was too small and encountered low temperature, it was not possible to operate properly.

As presented in Table 1 for 6 hives, it was impossible to take the bee sample for analysis. The reason was that inside the colony, there was almost no bee present. For the rest of the colonies, we analysed the infestation level with *Varroa* and the degree of infestation with *Nosema* spp. Based on the results, the level of infestation. Moreover, for all these hives, the Varroa mite infestation level was below 3%.

In Table 1, we have the hives with not enough bees for analysis marked in red. In yellow, we have all hives that were lost between January and February.
All hives lost between February and March were marked with purple. And in green, we have the hives that managed to enter the new season.

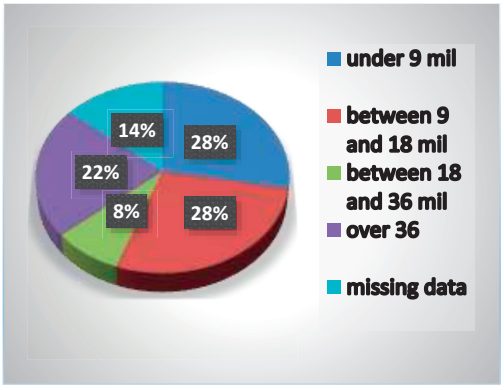


Figure 2. Spores distribution inside the sampled population

From the total population that remained in the winter, only 28% was under 9 million spores/bee. 58% of the population was over 9 million, and for the rest of 14%, we were unable to make the analysis. These values were interpreted based on the total number of hives presented in Table 1.

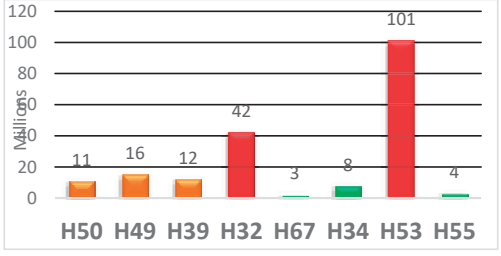


Figure 3. Number of spores/bee for the remaining hives

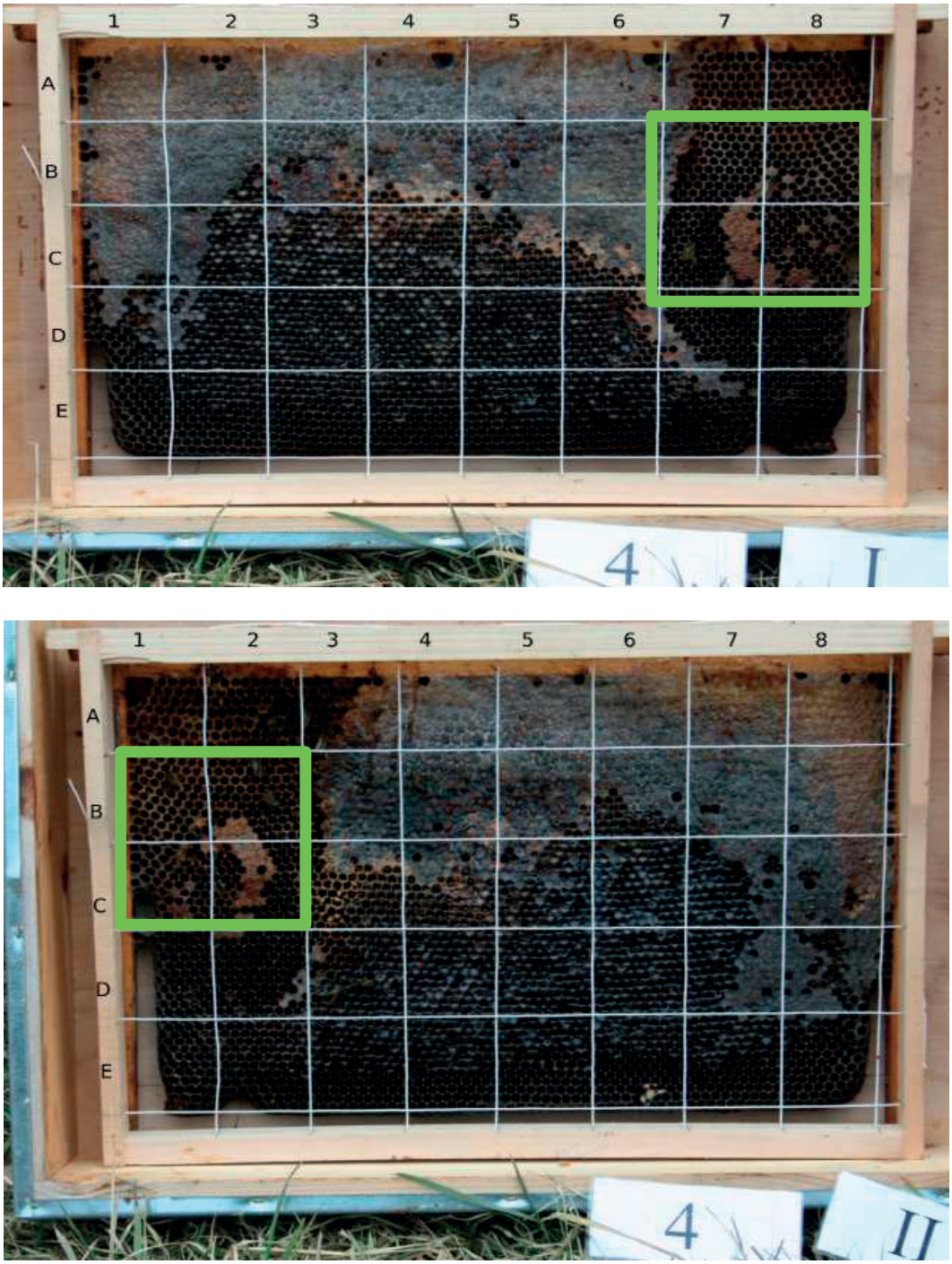
As shown in Figure 3, we have three colonies below nine million spores/bee marked in green. Three colonies that exceed 9 million spores but are under 20 million spores/bee marked with orange and two hives with an increased load of spores marked in red.
Despite the high *Nosema* spp. infestation level, Hive 32 and Hive 53 managed to go through the winter.

CONCLUSIONS

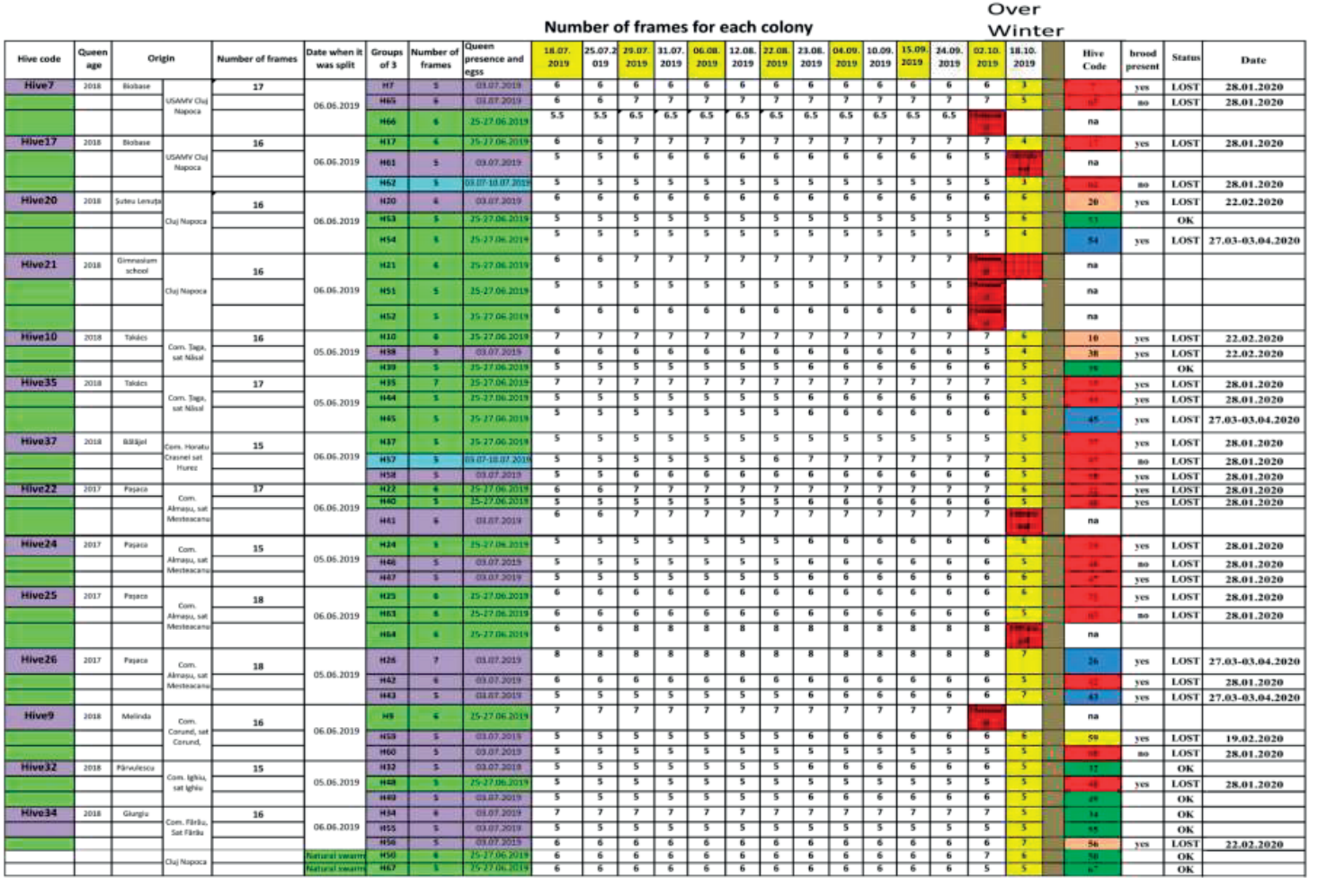
The breeding plan presents great potential. It can be adapted and applied at the apiary level as all the necessary equipment is usually available in an apiary focused on reproduction.
Based on our experience at the start of the breeding plan, all colonies should be inspected closely. Nosemosis can be triggered by different stress factors, including *V. destructor* infestation, prophylactic measures against *Nosema* spp. should be put in place.
Due to the hidden symptomatology of *Nosema ceranae* presence in Europe was confirmed; however, the exact arrival time is yet to be determined. Its presence was confirmed in more European countries, including Romania.
Due to its negative effects, active prophylactic measures should be put in place when breeding for resistance to *V. destructor.*
Trying to adapt against one non-native parasite puts a severe strain on the colony; fighting two at once will definitely lead to the colony's loss before it can adapt to the parasite.
Since over the active season and in the autumn, there were no clear signs of *Nosema* infestation and the level of infestation with *V. destructor* was relatively low, we suspect the presence of *Nosema crane*., further molecular analysis should confirm this.
Despite having a high mortality rate, all colonies that survived developed and responded to all the steps presented in the second season of the breeding plan protocol.

ACKNOWLEDGEMENTS

This research work was carried with support from the GRAL project. Nr. 162/2017, project code PN-III-P4-ID-PCE-2016-0637
Initiation of this protocol was possible due to the help of a few interested beekeepers who wished to participate and contributed with biologic material.
Special thanks to T. Blacquiere for early access to the breeding plan.
We do not take any credit for the development of this breeding plan. All the adaptations of this protocol were made only to be more suited to our local species of *Apis mellifera.* For more details about the breeding plan, please view Blacquiere et al. paper (2019).



Supplementary Figure 1.
Frame 4 side I and side II in 18.02.2020. Confirmation of brood presence in different stages.
Side I - capped and uncapped brood in section C7 and C8, and uncapped brood in section B7and 8.
Side II- Capped brood in section B2, C1 and 2. Uncapped brood in section B2 and C2. The presence of the brood is expected for this period. Food resources are present; however, the colony did not manage to survive.



Hive code	Queen age	Origin		Number of frames	Date when it was split	Groups of 3	Number of frames	Queen presence and eggs	18.07.2019	25.07.2019	29.07.2019	31.07.2019	06.08.2019	12.08.2019	22.08.2019	23.08.2019	04.09.2019	10.09.2019	15.09.2019	24.09.2019	02.10.2019	18.10.2019	Hive Code	brood present	Status	Date
Hive7	2018	Biobase	USAMV Cluj Napoca	17	06.06.2019	H7	5	03.07.2019	6	6	6	6	6	6	6	6	6	6	6	6	6	3	[illegible]	yes	LOST	28.01.2020
						H65	6	03.07.2019	6	6	7	7	7	7	7	7	7	7	7	7	7	5	[illegible]	no	LOST	28.01.2020
						H66	6	25-27.06.2019	5.5	5.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5	6.5	[illegible]		na			
Hive17	2018	Biobase	USAMV Cluj Napoca	16	06.06.2019	H17	6	25-27.06.2019	6	6	7	7	7	7	7	7	7	7	7	7	7	4	[illegible]	yes	LOST	28.01.2020
						H61	5	03.07.2019	5	5	6	6	6	6	6	6	6	6	6	6	5	[illegible]	na			
						H62	5	03.07-10.07.2019	5	5	5	5	5	5	5	5	5	5	5	5	5	3	[illegible]	no	LOST	28.01.2020
Hive20	2018	Suteu Lenuta	Cluj Napoca	16	06.06.2019	H20	6	03.07.2019	6	6	6	6	6	6	6	6	6	6	6	6	6	6	20	yes	LOST	22.02.2020
						H53	5	25-27.06.2019	5	5	5	5	5	5	5	5	5	5	5	5	5	6	[illegible]		OK	
						H54	6	25-27.06.2019	5	5	5	5	5	5	5	5	5	5	5	5	5	4	54	yes	LOST	27.03-03.04.2020
Hive21	2018	Gimnasium school	Cluj Napoca	16	06.06.2019	H21	6	25-27.06.2019	6	6	7	7	7	7	7	7	7	7	7	7	[illegible]	[illegible]	na			
						H51	5	25-27.06.2019	5	5	5	5	5	5	5	5	5	5	5	5	[illegible]		na			
						H52	5	25-27.06.2019	6	6	6	6	6	6	6	6	6	6	6	6	[illegible]		na			
Hive10	2018	Takács	Com. Taga, sat Năsal	16	05.06.2019	H10	6	25-27.06.2019	7	7	7	7	7	7	7	7	7	7	7	7	7	6	10	yes	LOST	22.02.2020
						H38	5	03.07.2019	6	6	6	6	6	6	6	6	6	6	6	5	4	[illegible]	38	yes	LOST	22.02.2020
						H39	5	25-27.06.2019	5	5	5	5	5	5	5	6	6	6	6	6	6	5	[illegible]		OK	
Hive35	2018	Takács	Com. Taga, sat Năsal	17	05.06.2019	H35	7	25-27.06.2019	7	7	7	7	7	7	7	7	7	7	7	7	7	5	[illegible]	yes	LOST	28.01.2020
						H44	5	25-27.06.2019	5	5	5	5	5	5	5	6	6	6	6	6	6	5	[illegible]	yes	LOST	28.01.2020
						H45	5	25-27.06.2019	5	5	5	5	5	5	5	6	6	6	6	6	6	6	45	yes	LOST	27.03-03.04.2020
Hive37	2018	Bălăjel	Com. Horatu Crasnei sat Hurez	15	06.06.2019	H37	5	25-27.06.2019	5	5	5	5	5	5	5	5	5	5	5	5	5	5	[illegible]	yes	LOST	28.01.2020
						H57	5	03.07-10.07.2019	5	5	5	5	5	5	6	7	7	7	7	7	7	5	[illegible]	no	LOST	28.01.2020
						H58	5	03.07.2019	5	5	6	6	6	6	6	6	6	6	6	6	6	5	[illegible]	yes	LOST	28.01.2020
Hive22	2017	Pașaca	Com. Almașu, sat Mesteacănu	17	06.06.2019	H22	6	25-27.06.2019	6	6	7	7	7	7	7	7	7	7	7	7	7	6	[illegible]	yes	LOST	28.01.2020
						H40	5	25-27.06.2019	5	5	5	5	5	5	6	6	6	6	6	6	6	5	[illegible]	yes	LOST	28.01.2020
						H41	6	03.07.2019	6	6	7	7	7	7	7	7	7	7	7	7	[illegible]	[illegible]	na			
Hive24	2017	Pașaca	Com. Almașu, sat Mesteacănu	15	05.06.2019	H24	5	25-27.06.2019	5	5	5	5	5	5	5	6	6	6	6	6	6	6	[illegible]	yes	LOST	28.01.2020
						H46	5	03.07.2019	5	5	5	5	5	5	5	6	6	6	6	6	6	5	[illegible]	no	LOST	28.01.2020
						H47	5	03.07.2019	5	5	5	5	5	5	5	6	6	6	6	6	6	6	[illegible]	yes	LOST	28.01.2020
Hive25	2017	Pașaca	Com. Almașu, sat Mesteacănu	18	06.06.2019	H25	6	25-27.06.2019	6	6	6	6	6	6	6	6	6	6	6	6	6	6	[illegible]	yes	LOST	28.01.2020
						H63	6	25-27.06.2019	6	6	6	6	6	6	6	6	6	6	6	6	6	5	[illegible]	no	LOST	28.01.2020
						H64	6	25-27.06.2019	6	6	8	8	8	8	8	8	8	8	8	8	[illegible]	[illegible]	na			
Hive26	2017	Pașaca	Com. Almașu, sat Mesteacănu	18	05.06.2019	H26	7	03.07.2019	8	8	8	8	8	8	8	8	8	8	8	8	8	7	26	yes	LOST	27.03-03.04.2020
						H42	6	03.07.2019	6	6	6	6	6	6	6	6	6	6	6	6	6	5	[illegible]	yes	LOST	28.01.2020
						H43	5	03.07.2019	5	5	5	5	5	5	5	6	6	6	6	6	6	7	43	yes	LOST	27.03-03.04.2020
Hive9	2018	Melinda	Com. Corund, sat Corund,	16	06.06.2019	H9	6	25-27.06.2019	7	7	7	7	7	7	7	7	7	7	7	7	[illegible]	[illegible]	na			
						H59	5	03.07.2019	5	5	5	5	5	5	5	6	6	6	6	6	6	6	59	yes	LOST	19.02.2020
						H60	5	03.07.2019	5	5	5	5	5	5	5	5	5	5	5	5	5	5	[illegible]	no	LOST	28.01.2020
Hive32	2018	Pârvulesca	Com. Iglița, sat Iglița	15	05.06.2019	H32	5	03.07.2019	5	5	5	5	5	5	5	6	6	6	6	6	6	5	32		OK	
						H48	5	25-27.06.2019	5	5	5	5	5	5	5	5	5	5	5	5	5	5	[illegible]	yes	LOST	28.01.2020
						H49	5	03.07.2019	5	5	5	5	5	5	5	6	6	6	6	6	6	5	49		OK	
Hive34	2018	Giurgiu	Com. Fărău, Sat Fărău	16	06.06.2019	H34	6	03.07.2019	7	7	7	7	7	7	7	7	7	7	7	7	7	5	34		OK	
						H55	5	03.07.2019	5	5	5	5	5	5	5	5	5	5	5	5	5	5	55		OK	
						H56	5	03.07.2019	6	6	6	6	6	6	6	6	6	6	6	6	6	7	56	yes	LOST	22.02.2020
			Cluj Napoca		Natural swarm	H50	6	25-27.06.2019	6	6	6	6	6	6	6	6	6	6	6	6	7	6	50		OK	
					Natural swarm	H67	5	25-27.06.2019	6	6	6	6	6	6	6	6	6	6	6	6	5	5	[illegible]		OK	

Supplementary Figure 2. Synthesis with development for selected hives

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

STUDY OF SOME PHYSIOLOGICAL AND BIOCHEMICAL PARAMETERS IN DAIRY COWS BRED AT DIFFERENT TEMPERATURE AND HUMIDITY REGIMES

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Abstract

The present study examines some physiological and biochemical parameters in dairy cows depending on the temperature-humidity index (THI) in spring (May) and summer (August) in two different time zones - 10 and 15h. The temperature-humidity index (THI) in a semi-open type of building, the body temperature, pulse rate and breathing were measured twice a day (10 and 15.00 o'clock), as well as the number of ructus of the dairy cows per 24 hours in spring and summer. Changes in the biochemical parameters of the total protein, creatinine and urea were ascertained in relation to the changes in THI and the season, which are within the reference values for cattle. The high THI lowers the blood sugar levels (up to 31%), but increases the total bilirubin levels (up to 3 times) and the transaminases activity (ASAT up to 3 and ALAT - up to 4.5 times) compared to the reference values for the respective species. The changes in THI values have a statistically significant influence on the heart rate, respiration and body temperature ($p<0.001$). The month (season) of the study has a statistically significant influence on the blood sugar, total protein, urea and bilirubin ($p<0.001$) and less influence on the body temperature ($p<0.01$) and creatinine ($p<0.05$.)

Key words*: dairy cows, physiological and biochemical parameters, season, temperature-humidity index (THI).*

INTRODUCTION

The cattle's ability to adequately respond to the high summer temperatures depends mainly on the production type and the breed. The dairy cows are more susceptible to heat stress than the meat breeds due to the fact that the former generate more metabolic heat. The highly productive cows are especially sensitive to the high temperature and this affects their blood profile (Hewett, 1974).

The high temperatures can disturb the normal physiological balance of the animal and lead to disorders in the water and protein exchange, energy, hormonal and mineral balance (Marai et al., 2000; Ivanova & Tasheva, 2020). According to Ordinance No 44/20.04.2006, the optimal temperature zone is 10-15ºC at a minimum of 5 and maximum of 28ºC. Temperatures above +18-20ºC are capable of causing heat stress to the highly lactating cows. Heat stress results from the imbalance between the ambient heat flow and the heat released from the body. The occurrence of heat stress is triggered by the high air temperature in combination with high or, the opposite, very low humidity. The comfort zone for the local cattle breeds varies between +4 and +20ºC, and for the highly lactating ones - between +9 and +16ºC. Each temperature increase above the optimum values leads to the activation of mechanisms connected to energy consumption and decrease in the adaptive abilities efficiency (Brown-Brandl & Tami, 2018). In the context of the globally altering climate, the heat stress is becoming a serious problem for the dairy cattle breeding. Mahdy et al. (2014) consider that the temperature- humidity index (THI) is one of the most important parameters reflecting the dairy cows overall comfort. The authors believe that the THI is a useful instrument in determining the heat stress occurrence. The temperature humidity index indicates the combined effect of the temperature and the relative humidity on the physiological, production, and other, parameters in cows. The plethora of studies show that the THI can be used as a heat stress indicator for dairy cows (Armstrong, 1994; Kadzere et al., 2002; Dikmen & Hansen, 2009; Hristev et al., 2020).

Mazzullo et al. (2014) prove that the environmental conditions are major stress factors influencing the animals and leading to serious disorders in their hematology-chemical and physiological parameters.

The blood indexes are sensitive to the changes happening in the organs and the cells even before the first clinical signs of a certain disease or a stress situation are exhibited. Therefore, according to Jain (1993), Otto et al. (2000), Ndlovu et al. (2007), Wood & Quiroz-Rocha (2010), the changes (deviations) in the different blood parameters values are indicators of an early organism response and may be used as a basis of early diagnostics, treatment and prevention of various pathological conditions.

The aim of the present study is to trace the changes in some physiological and biochemical blood parameters of dairy cows under the influence of different temperature-humidity regimes.

MATERIALS AND METHODS

The study was carried out in a Holstein-Friesian cattle breed farm situated in the region of Karnobat, South- East Bulgaria. The cows are bred free in separated boxes in a semi-open type of building and their feeding is unlimited with a total mixed ration. The cows in the farm are cooled by irrigation in the waiting zone of the milking area and by all-day-long ventilation in the barn during the warm months of the year. The study includes 24 cows which are the same in terms of period of calving. The monitoring of the physiological and biochemical blood parameters was performed in the spring (May) and in the summer (August). At the moment of the first examination (May), the cows are in their 1^{st} -2^{nd} lactating month, and during the second one (August)- respectively in 4^{th} – 5^{th}. The cows are in different lactation periods- from first to fifth. The heat stress level was determined by the temperature-humidity index (THI) which was measured with a Kestrel automatic measuring device. The THI recording was performed in the cows breeding area twice a day, at 10 and 15 o'clock, along with the measuring of the physiological parameters of each cow- pulse, rectal temperature and breeding intensity. The rectal temperature was measured with a digital thermometer in degrees Celsius. The breeding intensity was visually monitored by reporting the chest movement per minute, in accordance with the Zimbelman et al. (2009) method. Data regarding the activity in the rumen, expressed in the number of ructus per 24 hours, of each cow were taken from the farm management software. This activity was reported by straps, with microphones of the SCR by Allflex system fixed around the neck, which register each burp of the animals. The blood samples were taken once on the day of THI and physiological parameters of the cows reporting. The biochemical blood analysis was performed via Biomed ready-to-use tests. THI is incurporated in the models as an average of the values reported for the respective month- May and August, and displayed as an effect of the month. The data were statistically processed via SPSS-21.

The following model was used for the assessment of the controlled factors influence on the physiological parameters: $\mathbf{Y_{ijkl} = \mu + M_i + H_j + M{*}H_k + e_{ijkl}}$, where: $\mathbf{Y_{ijkl}}$ - dependent variable (each of the physiological parameters examined), $\boldsymbol{\mu}$ - mean effect, $\mathbf{M_i}$ - effect of the average THI for the month (classes), $\mathbf{H_j}$ -THI effect at the time of reporting, $\mathbf{M{*}H_k}$ - the related effect of the detection time in the month of reporting and $\mathbf{e_{ijk}}$ is the random residual effect. The THI effect on the ructus and the biochemical parameters examined is reported by using a single factor analysis $\mathbf{Y_{ij} = \mu + THI_i + e_{ij}}$, Where: $\mathbf{Y_{ijkl}}$ - dependent variable (each of the biochemical parameters examined and number of ructus), $\boldsymbol{\mu}$ e mean effect, $\mathbf{THI_i}$ - effect of the average THI for the month (classes) and $\mathbf{e_{ij}}$ is the random residual effect. The average of the least square (LSM) is calculated in fixed factors classes with the use of the analysis of variance (ANOVA) for the model.

RESULTS AND DISCUSSIONS

The THI values are reported twice a day for a period of 4 months (Table 1). The data exhibited shows that the average THI values are below 72 only in May. During the rest of the summer months the average THI values are above 74, and the lower values reported at 10 o'clock are bordering and almost 72-71.6 and

71.8, respectively. Armstrong (1994) presents a heat stress level in dairy cows classification depending on the THI values. The author states that there is zero stress in cows with values below 72, mild - from 72-79, temperate - from 80-89, severe - 90-98 and emergent above 98. According to this classification, the cows in the farm examined have been under mild to temperate heat stress during the daytime of the summer months from June to August. The results of our studies show that only the THI values reported at 10 o'clock in May are within the heat comfort zone. The THI values at 15 o'clock and those in both time frames in August are above 72 which comes to indicate that the cows experience mild to temperate heat stress during almost the entire daytime irrespective of the ventilators working at the same time. In a one-year study of the climatic conditions in South Bulgaria, Dimov et al. (2017) ascertain that the average daily THI values of above 75 reported in the summer provide conditions for heat stress in the dairy cows bred in semi-open type of buildings.

Table1. Average values and variation of THI in months of reporting

Reporting month	THI		
	x ± SE	min	max
May	71.72±0.30[a]	69.3	74.2
June	75.06±0.26[b]	71.8	81.5
July	74.32±0.16[c]	71.6	77.6
August	77.35±0.33[d]	74.5	81.5
Average	74.62±0.16	69.3	81.5

Note: The differences between the values indicated with the same letters are significant only upon P<0.05.

There is a certain risk of similar conditions during the spring, too, when the average daily values are above 69. Analogous results are also reported by Hristev et al. (2020). This proves the observation that the registered temperatures and humidity in both seasons might be the reason for temperature homeostasis disorders in cows during the daytime. According to Grant (2009) the indexes values are not fixed but change throughout the day. The temperature homeostasis maintenance in dairy cows is possible upon THI values of up to 70 when the thermoregulation mechanisms function normally and allow the maintenance of a normal body temperature (Kadzere et al., 2002). Therefore, most of the researchers consider the index 72 as corresponding to temperature of 25ºC and relative humidity of 50%, and the index values between 77 and 87 as critical. The lethal cases in cows increase when the values are above the aforementioned ones (Vitali et al., 2009). Many authors point that the short- term (several-day) high values of THI do not have such a serious effect as the month-and-more-continuing influence of THI values associated with heat stress, especially on the biochemical, production and reproduction parameters of dairy cows (Silanikove, 2000; Bouraoui et al., 2002; Kadzere et al., 2002; Spiers et al., 2004; Chase, 2006). Taking these notions into account, the biochemical blood parameters of the cows were reported in August- after a two-month influence of conditions with THI daily values predisposing to heat stress levels (mild to moderate) and compared with the biochemical status under conditions without heat stress in May. Due to the fact that the cows included in the study are in the same stage of lactation and are bred and fed under the same conditions, the main factor is THI, and the month of study effect overlaps with that of the THI. The physiological parameters and THI are reported twice a day at the same time - at 10 and 15 o'clock, with the exception of the ruminations index. It is displayed as a daily average and it is impossible the THI effect at 10 and 15 o'clock to be reported. The analysis of variance (Table 2) shows that there is a considerable effect of THI at the time of reporting ($P<0.001$) and the related effect THI for the month and time of reporting ($P<0.01$) on the pulse of the animals. When it comes to the respiratory movements, significance is reported only with reference to the related effect of THI for the month and hour of reporting ($P<0.001$). Both the average THI value for the month of study ($P<0.01$) and the THI at the time of reporting ($P<0.001$) but not the related effect of the two factors influence the rectal temperature. No considerable influence of the THI values for the month of examination is reported regarding the average daily ructus values. Table 3 displays the results regarding the THI influence on the physiological parameters examined.

It is noticeable that the pulse frequency during the spring and the summer is either at the upper physiological borderline or exceeds it in both time intervals. The monitoring of the frequency

and the properties of the pulse provides additional information about the heart rate, the blood vessels condition as well as about the blood circulation as a whole.

Table 2. Analysis of variance on the influence of the controlled factors on the physiological parameters examined

Parameters	Number of reports n	Mean THI per month		Reporting time (THI)		Mean THI per month *Reporting time (THI)	
		MS	F P	MS	F P	MS	F P
Puls n/min	24	0.75	0.329 -	30.08	13.193***	18.75	8.223**
Breath n/min	24	320.33	3.96 -	147.00	1.816 -	1587.00	19.607***
R. temper C^0	24c	1.47	12.30**	2.00	16.8***	0.27	2.3 -
Ructus n/24 h	12	11.48	0.375	-	-	-	-

Note: F - Fisher's criterion ***P<0.001; **P<0.01; *P<0.05; - no significance.

Table 3. LSM values of the physiological parameters depending on the average THI for the month and time of reporting

Parameters	Number of reports	Mean THI per month		Reporting time		May		August	
		May THI=71,75	August THI=78,30	10 o'clock THI=72,85	15 o'clock THI=77,20	10 o'clock THI=69,30	15 o'clock THI=74,20	10 o'clock THI=76,40	15 o'clock THI=80,20
	n	LSM ± SE	LSM ± SE	LSM ± SE	LSM ± SE	LSM ± SE	LSM ± SE	LSM ± SE	LSM ± SE
Puls n/min	24	20.92±0.31	20.67±0.31	20.00±0.31	21.58±0.31	20.75±0.44	21.08±0.44	19.25 ±0.44	22.08±0.44
Breath n/min	24	40.0±1.83	45.17±1.83	40.83±1.83	44.33±1.83	44.0 2.60	36.0±2.60	37.66±2.60	52.67±2.60
R. temper C^0	24	38.07±0.07	38.72±0.07	38.34±0.07	38.75±0.07	38.24±0.10	38.50±0.10	38.44±0.10	39.00±0.10
Ructus n/24h	12	555.0±15.97	541.17±15.97						

The change of pulse is heavily influenced by the THI values (Table 3), the time of measurement has a weaker effect, while the month does not affect this parameter at all. According to Atkins et al., (2018) the breathing may be considered a more precise criterion than the different indexes measuring the heat stress. This parameter may vary in wide ranges depending on whether the cow is in standing or lying position, its productivity and other factors. The momentary reporting, however, should not be always regarded as a norm due to the fact that the animal might have been stressed from the manipulation itself. The results of our study show a significant increase in the breathing rate during the 15 o'clock measurement in August with values almost twice as high as the upper reference value.

The accelerated lungs ventilation leads to a decrease in the carbon dioxide levels which causes disorder in their balance with the blood bicarbonates and the pH levels in the organism. According to Brouk (2003), upon increase in the outer temperature above 21.2^{O}C, the evaporation cooling becomes the main method for releasing heat from the body. Regardless of the double- accelerated breathing of the cows examined, their body temperature remains within the reference values (38-39^{O}C). Similarly to the pulse, the breathing rate and the body temperature are dependent on the THI ($p<0.001$). The month and the time of examination have a temperate to strong influence on the body temperature ($p<0.01$) but small on the breathing. Upon heat load increase, the breathing rate also increases, the food consumption decreases and the water intake rises (Bernabucci et al., 2010). Along with the pulse rate, the rectal temperature also goes up (Avendono-Reyes et al., 2012). The body temperature, however, is unstable. Its values change throughout the day in relation to the metabolic processes and the thermoregulation abilities. Therefore, the THI can be considered the major factor influencing both the heat production and heat release. The rumination monitoring may be used for assessment of the feeding results, the breeding conditions and the cows health. Through the ructus cows release gases formed in the forestomach as a result of the fermentation processes happening there. The accumulated gases are released as a reflex following the activity of the reticulo-rumen and the abdominal contraction. The ructus rate depends on the speed and the degree of gas formation in the forestomach. This is connected to the food content, microbiological and chemical processes in the rumen. Their number in cows ranges from 15 to 90 per hour (Gabrashanski et al., 1989). No influence of the THI, month and time of examination on the rumination was

ascertained. According to Bernabucci et al. (1999), upon heat stress, the initial reaction observed is decrease in the rumen movement and the ructus, but upon its continued influence, the animals tend to acclimatize and the ruminations are recovered and reach their levels of prior the heat stress. This is also what we observed upon gathering our data for the months May and August. The acclimatization is a process during which the animals adapt to the environmental conditions triggering behavioral, hormonal and metabolic changes which allow the organism to survive in the new 'physiological condition'.

Table 4. Analysis of variance of the influence of the THI average values on the blood biochemical parameters

Parameters	Number of reports n	Mean THI per month	
		MS	F P
Glucose mmol/L	12	6.33	22.44***
Total protein g/L	12	836.4	20.58***
Creatinine µmol/L	12	100.0	1.98 -
Urea m 45 mol/L	12	18.38	43.20***
Total bilirubin µmol/L	12	878.22	30.11***
ASAT U/L	12	66.7	0.13 -
ALAT U/L	12	170.67	1.35 -

Note: ***P<0.001; **P<0.01; *P<0.05; - no significance.

This load which is provoked by the heat in an attempt to maintain the homeostasis affects the lipid and protein metabolism, leads to disorders in the liver functioning, causes oxidative stress, endangers the immune response and decreases the reproduction and productive parameters. The analysis of variance performed indicates a noticeable effect (P<0.001) of the THI average values for the respective month of examination on the values of the following blood biochemical parameters- Glucose, Total protein, Urea and Total bilirubin. No significant effect of the THI values on the Creatinine, ASAT and ALAT values is reported. Table 5 displays the LSM values of all biochemical blood parameters examined irrespective of the THI effect.
The blood sugar is one of the main biochemical parameters characterising the carbohydrate exchange. Its levels in ruminants are low which is connected to their specific exchange (Gromyko, 2005). The glucose levels in the blood in August are 34% lower than those in May. Similar conclusions were reached by Gorski & Saba (2012) under an analogous experiment.

Table 5. LSM values of the blood parameters depending on the THI average values for the month of reporting

Parameters	Number of reports n	Mean THI per month	
		May THI=71.75 LSM ± SE	August THI=78.30 LSM ± SE
Glucose mmol/L	12	3.00±0.195	1.97±0.094
Total protein g/L	12	76.27±2.09	88.07±1.54
Creatinine µmol/L	12	88.42±1.94	84.33±2.49
Urea mmol/L	12	2.96±0.23	4.71±0.13
Total bilirubin µmol/L	12	6.68±1.22	18.78±1.84
ASAT U/L	12	117.42±8.60	120.75±1.37
ALAT U/L	12	26.58±4.38	31.92±3.25

As it was underlined, the blood sugar levels are not the main source of energy for the ruminant organisms, however, at the end of the pregnancy and the beginning of the lactation, a big part of it is used for lactose and milk fat synthesis, and therefore, its levels are indicative upon some pre-pathological and pathological conditions. Darul & Kruczynska (2005) point out that the blood sugar levels decrease after birth and initiation of active lactation due to the change in the energy balance of cows. Due to the rising decrease in the fatty acids oxidation upon chronic heat stress, the stressed animals become more and more dependent on the glucose for their energy needs. When taking the reduced feed intake into account, it can be seen that there has been a discrepancy between the energy received and that consumed upon lactation and metabolism. (Baumgard & Rhoads, 2007). Subsequently, the cows subjected to moderate heat stress (Moore et al., 2005) are in a negative energy balance condition. The results indicated in table 5 show that the blood sugar levels are influenced by both the THI and the season (p<0.001). The levels of the total protein reported in May are within the reference values, but those in the summer tend to increase. The hyperproteinemia combined with hypoglycemia are probably a sign of a threatened ketosis and early stages of liver damage. The feeding is the factor which has a major influence on the protein levels in the blood. They are also affected by the liver, kidneys, gastro-intestinal system condition, the stress, water loss, and so on. In their efforts to improve the dairy cows productivity, farmers increase the proteins in the blood at the expense of the crude fibre content, which affects both the rumen homeostasis and the blood protein levels. Our study ascertained a reasonable THI

influence (Table 5) on the albumin levels ($p<0.001$). The changes in the blood urea levels are primarily related to the functional condition of the liver. A bigger part of the proteins contained in the feeds are hydrolised to amino acids, and after their degradation, the extra ammonia is absorbed in the blood, then goes into the liver and turns into urea (Holodov & Ermolaev, 1988). The results which we achieved show that the ammonia levels in the blood in May are below the reference values for the respective type of animals. In August the levels rise to 44% but are still within the reference values. During pregnancy, all metabolic processes activate to meet the growing needs of the fetus and the cow. The increased urea levels show a high degree of protein feeds assimilation (Holodov & Ermolaev, 1988). Our studies display that the urea levels are statistically influenced by the THI ($p<0.001$). Creatinine, along with the urea, is a product of the protein exchange. It is formed during the metabolism in the muscle tissue and is excreted by the kidneys. Table 5 displays the effect of the THI on the creatinine levels. The average levels after all tests are within the reference values for the respective kind. It is only the season that reasonably affects the creatinine levels ($p<0.001$) (Table 5). Taking the creatinine levels into account, we can make an assessment of the kidney excretory functions and its metabolism speed in the muscle tissue of the dairy cows. The heat stress mostly affects the organs with high metabolism speed-liver, kidneys and the epithelium layer of the digestive tract mucous membrane. This is due to the redistribution of the blood towards the skin. In this case the internal organs experience nutrient deficiency and accumulate metabolism products whose free radicals damage the organs cell walls. The bilirubin levels in the blood serum of the healthy cattle are negligible. They are often increased after calving or during food deprivation. Its excretion is used for the diagnosis of steatosis in cows. The bilirubin concentration in our studies displays an upward trend from 6.7 in May to 18.8 in August. These values are very high and must be accompanied by severe icterus or acetonaemia which were not observed. Probably they were the result of a mass invasion of haemosporidia which are going to be a subject of a future study. Table 5 shows that the bilirubin levels are reasonably affected by the THI and the season ($p<0.001$). The metabolic processes in the animal organisms speed up in conditions endangering the maintenance of the organism homeostasis. The enzyme systems play a huge role in these processes. The enzyme levels are one of the fast-reacting units of the biochemical homeostasis and reveal even the slightest changes in the metabolism of the animals, they help for the identification of pathological processes prior the display of clinic signs (Yarovan & Novikova, 2012). The ASAT and ALAT enzymes are important for the amino acids metabolism as they catalyze the transfer of amino-groups to the keto acids. They are present in all organs and tissues and so their increased activity in the blood serum is indicative with reference to many diseases (Kazartsev & Ratoshny, 1986). The results of the present study show a drastic increase in both transferases. The average ASAT levels in May are 117 U/L, and 121 U/L in August, and the ALAT levels are respectively 27-32 U/L. No significant THI influence is indicated due to its low variation. Moore (1997) came to the conclusion that the increased ALAT serum levels are usually indicative of liver damage in the milk cattle. The hepatocytes are highly sensitive upon degenerative processes, and therefore, the ALAT plasma activity increase is rarely an indicator with a clinical importance unless it is twice the upper reference values, as is the case. The increased ASAT (three times the reference value!) must be carefully considered because this enzyme is contained not only in the liver but also in other tissues (heart and skeleton muscles, the brain, kidneys, pancreas and the lungs). The slighter ALAT increase is due to the fact that a big part of the enzyme is in the mitochondria and is excreted only upon severe degenerative cell damage. According to one of our previous studies (Ivanova & Tasheva, 2020), the high transaminase levels might be a result of the high productivity of these animals, which is the so called productive stress (Cozzi, 2011) The ASAT and ALAT activities are accelerated even upon moderate heat stress when there is an accelerated lactation and metabolism (Gorski & Saba, 2012).

CONCLUSIONS

The changes ascertained in the biochemical parameters of the total protein, creatinine and urea related to the changes in the THI and the season are within the reference values for cattle.

When the THI is high, the blood sugar decreases (up to 31%), the total bilirubin levels increase (up to 3 times), and the transaminase activities also go up (ASAT up to 3 and ALAT-up to 4.5 times) the reference values.

The THI changes statistically affect the pulse rate, breathing and the body temperature ($p<0.001$).

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

EFFECTS OF HORNS ON PRODUCTION AND REPRODUCTION EFFICIENCY IN ROMANIAN BLACK AND WHITE DAIRY COWS - PRELIMINARY STUDY

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Abstract

The widely growing interest in animal welfare has placed many livestock production practices such as disbudding or dehorning, under enhanced scrutiny. Disbudding is a commonly applied procedure that eases the management of cattle, having welfare implications given that the integrity of the animal is impaired. Aim of this study was to evaluate the effects of horns on production and reproduction efficiency and welfare of dairy cows. A total of 34 Romanian Black and White cows managed under identical conditions, were either horned (n = 17) or polled as a result of disbudding as calves (n = 17) and kept mixed, being housed in tied stanchion barns. Horns presence significantly influenced ($p \leq 0.05$) body condition score, with horned cows having higher fat deposits and maintaining better condition during lactation, compared to polled animals. However, no significant influence ($p \geq 0.05$) of horns was found on fertility traits, coat cleanliness, mastitis and retained placenta incidence or integuments alterations.

Key words*: animal welfare, calves disbudding, cattle dehorning, dairy cattle, polled cows.*

INTRODUCTION

During natural selection horns have provided advantages concerning defence and competition for resources (Knierim et al., 2015). Throughout the last decade the selection of cattle shifted towards practices such as polledness, disbudding representing a widely accepted welfare concern, given that animals experience high levels of pain (Gavojdian et al., 2018). Throughout history, presence of horns was appreciated and included in selection (especially in heritage breeds such as Hungarian or Italian grey breeds), given the additional advantage that age of the animal can be estimated by counting the surface rings (Knierim et al., 2015). Dehorning is defined as the removal of horns in older animals, whereas disbudding is generally defined as removal of horns in calves of up to 2 months of age. Surgical treatment such as dehorning is welfare relevant for various reasons (Winckler et al., 2002). Calves disbudding or cattle dehorning are regarded as a common and undesirable procedure in dairy farming, since that, in horned cows head butts and fast head movements are a risk of injuries for other herd mates and also for the stock-people (Cozzi et al., 2015). Nowadays, almost all dairy cattle are dehorned as calves to avoid injuries (Windig et al., 2015). The phylogenetic functions of horns in cattle can include a benefit for males, regarding competition for mates under natural selection (Bro-Jorgensen, 2007), moreover, horn size was showed to express health and fitness in African bovines (Ezenwa and Jolles, 2008). However, these phylogenetic functions are not relevant under the current livestock systems, given the wide use of artificial inseminations in dairy cattle and the single bull mating system used in beef production. Dehorning was showed to cause behavioural, physiologic and neuroendocrine changes in cattle (Stock et al., 2013). Up-to-date, more than 80% of the European dairy cattle are dehorned or disbudded, in most cases without the use of pain relief medication (De Boyer des Roches et al., 2014), this being a clear animal welfare issue affecting the cows integrity. A reliable and feasible solution could be

represented by the introgression of polledness genes from Aberdeen Angus breed to specialised dairy breeds (Windig et al., 2015). The alternative is to breed polled cattle that do not develop horns and therefore do not require to be dehorned (Prayaga, 2007; Gotz et al., 2015). A lower percentage of cattle reared in tie-stall systems, compared to loose housing systems are dehorned (Cozzi et al., 2015).

Significant efforts for artificial selection were made for the reduction of horns frequency, with a special focus on the use of genetically polled cattle strains. This is limited given the lower number of polled AI bulls available commercially and restraints of farmers to use bulls that have generally lower estimated breeding values (Knierim et al., 2015). Given that, polled Holstein-Friesian derived breeds were shown to have lower average genetic merit than their horned contemporaries (Cole et al., 2019). However, previous studies on breeding programs to produce genetically polled bulls have been successful in the Fleckvieh (Gotz et al., 2015) and the Charolais breeds (Windig et al., 2015).

The overall objective of this preliminary study was to evaluate the effects of horns on production and reproduction efficiency and welfare of dairy cows housed in tied stanchion barns, when milk yield attributes and reproduction outputs are concerned.

MATERIALS AND METHODS

Animal management

The preliminary study was performed at the Research and Development Institute for Bovine Balotesti (44°36′46″N 26°4′43″E) Romania, (altitude of site 92 m), where 34 (17 horned and 17 polled) purebred Romanian Black and White cows (Holstein-Friesian group, Bălţată cu Negru Românească national name) were raised under identical conditions. Cows taken into study were between 1th and 4th lactation, during the summer of 2020. Animals were kept in a tie-stall barn (170/85 cm), using wheat straws as bedding, having *ad libitum* access to water, mineral blocks and during warm weather had access to outside paddocks (8 m^2/head, maximum 12 hours/day). The feeding line outside was not individualized, cows competing for access to feed, ensuring minimum 0.75 cm/head. Cows were milked twice per day in the barn (starting at 5:00 and 17:00), with individual milking machines and received a daily feed ration of 35 kg corn silage, 6 kg of alfalfa hay and 7 kg of concentrates. Concentrates were fed exclusively inside the barn, after milking. Dehorning was carried out at the age of up-to two months, only on 17 female calves. The two groups were formed through unrandomized selection from the herd, with age, weight and parity balanced. A data-set from 34 animals, with 13 parameters per cow was taken into account and analysed for estimation of the effects concerning the presence of horns on production and reproduction outputs.

Data collection and statistical analysis

Milk yield per milking session (kg) and milk duration (minutes) together with ID tag number, were collected directly by one observer in the barn during the milking procedure. The average milking speed (kg/min) was obtained by fractioning production to time spent milking. Milk production per lactation was taken from the results of the official performance recordings, and standardized for the first 100 days in milk (100 DIM) and mature equivalent (cow's parity), using correction coefficients (Cziszter et al., 2016). Body condition score (BCS) was recorded on the same day, using a scale from 1 (severe under-conditioning) to 5 (severe over-conditioning) in increments of 0.5 (Kock et al., 2018). Cleanliness of udder, rump and hind legs (scores 0 - no dirt or minor splashing, 1-intermediate or 2 - separate or continuous plaques of dirt) were evaluated for each individual cow according to WelfareQuality® (2009) protocol for dairy breeds. The body weight of cows was measured using a weight tape. Reproductive outputs of the cows were recorded by the research institute's veterinarian and technicians. Worth mentioning is that the calving interval (CI) was calculated as the difference in days between the last lactation and the start of the penultimate. Mastitis and retained placenta incidences were recorded from the experimental farm health registers, while the tarsal joint lesions incidence was

evaluated during the horn presence assessment of animals. All the statistical inferences were carried out using comparisons between the 2 horn classes (phenotypes) using Minitab software (Minitab LLC®). Decisions about the acceptance or rejection of the statistical hypothesis have been made at the 0.05 level of significance

RESULTS AND DISCUSSIONS

The phenotype of cows significantly influenced body condition score (p-value = 0.017), with the horned cows having higher fat deposits, compared to polled cows. However, no statistically significant influence was found on milk yield (p-value = 0.782), body weight (p-value = 0.809) or milking speed (p-value = 0.863) (Table 1).

Current results regarding the influence of horned/polled phenotype on body condition score (BCS) might be attributed to the fact that polled cattle are generally regarded to be less aggressive when competing for feed and resources, compared to their horned counterparts. Similar to our findings, Knierim et al. (2015) reported a decreased risk of injury in polled cows, this being corelated with a lower social position in horned-polled mixed herds.

On the contrary, Gavojdian et al. (2018) found no correlations between phenotype classes and BCS in Fleckvieh dual-purpose cows.

Milk yield and milking speed were not influenced by the phenotype (p>0.05). Current results are not in accordance with those published by Gehrke et al. (2016) and Gavojdian et al. (2018), which reported that polled German Holstein and Fleckvieh dual-purpose cows had lower milk yields than their horned contemporaries, both authors attributing the differences to the social hierarchy of cows. Alongside the lower milk yield of the dairy cows, Dressel et al. (2016) found that polled German Holsteins bulls had lower breeding values for milk yield.

The body weight of cows was not influenced (p>0.05) by the horned phenotypes. Previous studies outlined that in hornless herds, body weight is the main influencing parameter concerning social rank (Lanaeta-Hernandez et al., 2013), while in horned herds, cows age and experience are the main influencing factors (Knierim et al., 2015). Holand et al. (2004) found a correlation between body weight and social rank in polled herds.

Lack of differences in the parameters of cows from the two phenotypes in the current study could be explained by the feeding regime under tied stanchion barn, where competition for feed was significantly reduced between the individual cows. Moreover, Windig et al. (2015) reported that horned animals are better adapted for the tie stalls and it seems more practical to reintroduce polledness phenotypes only in loose housing systems. Several studies have shown that polled and horned cattle have similar genetic merit for calving ease, health traits, growth rates, and reproduction traits, thus results from previous studies were made both on horned/dehorned cows and horned/genetically polled animals (Lamminger et al., 2000), with no differences among them.

Table 1. Milk yield, body weight, body condition score (BCS) and milking speed in horned and polled cows (mean ± SEM)

Phenotype	Milk yield (kg/100 DIM)	Body weight (kg)	BCS (1-5)	Milking speed (kg/min)
Horned	3195.0 ± 194.00[a]	688.7 ± 29.00[a]	3.02 ± 0.24[a]	1.58 ± 0.162[a]
Polled	3271.0 ± 182.00[a]	691.5 ± 23.20[a]	2.17 ± 0.205[b]	1.59 ± 0.136[a]

[1]DIM=days in milk; [2]SEM - standard error of the mean; [3]Column means with different superscript differ significantly at p≤0.05.

Regarding the effects of horns on reproduction efficiency, no significant influence of the phenotype (horned/polled) was found for the following parameters: number of inseminations per gestation (p-value = 0.782), calving interval (p-value = 0.733), age at first calving (p-value = 0.986) or retained placenta incidence (p-value = 0.389) (Table 2).

Number of inseminations per gestation (AI) was not influenced (p>0.05) by the horned

phenotype in our study. Current results are in accordance with those previously published by Gavojdian et al. (2018), which found no correlations between horn status and the number of AI/gestation in dual-purpose cattle. There are previous reports in the un-scientific literature that polled dairy cattle have reduced fertility compared with their horned counterparts (Cole et al., 2019). Calving interval was not influenced by existence/absence of the horns ($p>0.05$), however, the calving interval was shorter with 70 days in horned cows, when compared to polled animals. Age at first calving was not influenced by the horn phenotype of cows ($p>0.05$) nor did the retained placenta incidence ($p>0.05$), although, polled cows had higher incidences of retained placenta, when compared to horned animals.

Table 2. AI/gestation, calving interval, age at first calving and retained placenta in horned and polled cows (mean ± SEM)

Phenotype	AI/gestation	Calving interval (days)	Age at first calving (months)	Retained placenta (%)
Horned	3.0 ± 0.48^{a}	472.6 ± 31.60^{a}	28.3 ± 1.43^{a}	5.8 ± 5.88^{a}
Polled	2.2 ± 0.36^{a}	542.5 ± 59.80^{a}	28.5 ± 1.09^{a}	23.5 ± 10.60^{a}

[1]AI = artificial inseminations; [2]SEM - standard error of the mean; [3]Column means with different superscript differ significantly at $p \leq 0.05$.

No significant influence of the horn phenotype was found on integuments alterations, such as the tarsal joint lesions incidence (p-value = 0.389), mastitis incidence (p-value = 0.782), cleanliness of udder (p-value = 0.406), cleanliness of rump (p-value = 0.828), cleanliness of hind legs (p-value = 0.750) (Table 3).

Tarsal joint lesion incidence was not influenced ($p>0.05$) by the phenotype horn status. Pilz et al. (2006), cited by Knierim et al. (2015), reported that some farmers believe that polled animals tend to have a higher lameness incidence, which could be linked with the tarsal joint lesions. Mastitis incidence was not influenced ($p>0.05$) by the phenotype, although polled cows expressed a higher mastitis incidence, this might be attributed to udder injuries caused by the horn thrusts. Knierim et al. (2015) found some consequences regarding horn thrusts, which translates into visible blood traces in milk, having further economic implications. Cleanliness of the rump, udder and of hind legs were not influenced ($p>0.05$) by the phenotype (horned/polled). Numerous studies stated that horns may be used during self-grooming of cow body regions which are otherwise out of range (Knierim et al., 2015).

Further studies, on a greater number of animals and a more diverse range of rearing systems is advised, especially in organic production registered dairy farms, where removal of horns is not recommended and has a low level of acceptance among the consumers and policy-makers.

Having in mind the current results, keeping mixed herds of horned-polled adult cows is not advisable, given the social hierarchy structure of cattle and the intense competition of the animals for resources (feed, resting space, water, shade, etc.). Moreover, horns presence is expected to have a greater impact in loose housing systems and there where the stocking density is high, compared to the current setting, where cows were fed their concentrates indoors, and the competition for feed was significantly reduced.

Table 3. Tarsal joint lesion, mastitis incidence cleanliness of rump, cleanliness of the udder and cleanliness of hind legs in horned and polled cows (mean ± SEM)

Phenotype	Tarsal Joint Lesion (%)	Mastitis (%)	Cleanliness of rump (0-2)	Cleanliness of udder (0-2)	Cleanliness of hind legs (0-2)
Horned	23.5 ± 10.60[a]	23.5 ± 10.60[a]	1.37 ± 0.183[a]	1.14 ± 0.143[a]	1.33 ± 0.142[a]
Polled	41.2 ± 12.30[a]	29.4 ± 11.40[a]	1.30 ± 0.133[a]	1.40 ± 0.163[a]	1.41 ± 0.149[a]

[1]SEM - standard error of the mean; [2]Column means with different superscript differ significantly at p≤0.05.

CONCLUSIONS

Current partial results suggest that horned animals achieve greater fat deposits and are maintaining better condition throughout the lactation, compared to polled animals, when kept under identical feeding and management conditions. Breeding genetically polled cattle is a viable alternative, providing a long-term solution to the presence of horns issues and addressing the welfare concerns of dehorning and disbudding of calves and cattle.

However, production and reproduction efficiency parameters were not influenced by the presence/absence of horns in dairy cows managed under tied stanchion barn.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

DETERMINING THE CURRENT STATE OF CURL'S IMPROVEMENT IN STRENGTH AND ELASTICITY

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Abstract:

The purpose of the research was to determine the level of improvement in skin production and how to express the strength and elasticity of curls. The importance of these characters is essential because both participate in shaping a certain type of curl that will have the quality of keeping the main shape and characteristics for a longer time. The biological material subject to appreciation belongs to the Karakul of Botoșani breed located in the Principal Class of the Genealogical Register. The experimental protocol was based on the assessment of the characters in three consecutive seasons and aimed at determining the degree of improvement of the curl for several selected characters. Based on the results, it can be concluded that the black variety average score is close to the maximum, thus indicating that the improvement of these characters is in a more advanced phase. Determining the degree of significance between the average scores for the gray and pink varieties are significant for P<0.01 and still significant for P<0.05 between pink and grayish.

Key words*: curl, Karakul sheep, loop, pelts.*

INTRODUCTION

Strength and elasticity are selection characters because they participate in expressing the quality of the curl in the skins of Karakul of Botoșani lambs. Both characters are influenced by the height, degree of closure of the curls, the density and quality of the fibers. Also, both the elasticity and the resistance of the curl can be influenced by the density of the curls as well as the surface of the skin.

Being characters that are evaluated in lambs in the first neonatal period, especially by subjective methods, their improvement will be strongly influenced by the technique and degree of training and objectivity of the staff involved in establishing productive performance in the Karakul of Botoșani breed.

So, in the production of skins, the great shortcoming that could make it difficult to obtain the expected effect of the selection is due to the fact that many of the characters followed in the selection are evaluated in practice using mainly subjective methods (Anonymous, 1982; Pascal, 2007; Pascal et al., 2010). For this reason, the results of evaluations are often inaccurate and inconsistent (Schoeman, 1968; 1969; Albertyn et al., 1990, quoted by Schoeman, 1998 and Pascal, 2011) and the clear determination of the effect due to a character evaluated in this way is very difficult to quantify.

Both strength and elasticity depend largely on the quality of the component fibers. The inclusion among the selection criteria and the properties on which the quality of the fibers depends is justified by the fact that all these influence the durability and resistance. The quality of the fibers is determined by the diameter of the fibers and the shape and arrangement of the cells at the level of the cuticular layer. Based on these findings, most researchers in the country and abroad (Taftă et. al., 1998; Pascal, 2007) state that a good quality of the hair coat is associated with a fiber length

between 9 and 12 mm and an average thickness of 30-33 μm.

MATERIALS AND METHODS

The biological material analyzed was represented by Karakul of Botoșani lambs of several color varieties, obtained over three consecutive generations at the herd located at the Research and Development Unit for Breeding Sheep and Goats Popăuți - Botoșani.
In each season of assessment of the characters that represented the main objective of the research performed, the working method used was based on the technical norms specified in Section 1.4 and 1.5 of the MADR Order no. 22/ 20.01.2006, published in the Official Gazette of Romania no. 146 of 15.02.2006 and in which are specified the aspects based on which the official Control for the skin production obtained from Karakul is performed.
All lambs obtained were subjected to skin quality assessments in the first two days after lambing, regardless of variety or condition of the curl at 24 hours after birth.
In this sense, the experimental protocol established the development of several activities and objectives, different in terms of implementation, for the analysis of the practical and technical impact on the reproductive activity applied to the Karakul of Botoșani breed.
All lambs obtained were subjected to skin quality assessments in the first two days after lambing, regardless of variety or condition of the curl, and took place in three consecutive campaigns, corresponding to the lambing season 2018, 2019 and 2020.
The working method used in the evaluation was based on the use of subjective methods for immeasurable characters and laboratory determinations for quantitative characters.
The data obtained were compared with those obtained in the performance control of the first generation of lambs obtained from the establishment of the Genealogical Register, respectively the generation of lambs from 2005.
The statistical processing of the data was based on the use of computer software SAVC (Statistical Analysis of Variance and Covariance 2003). To test the statistical significance of the differences between the averages parameters values studied and the correlations between them, the variables analysis (ANOVA Single Factor) and the Pearson Correlation algorithms were used, both included in the computer program used.

RESULTS AND DISCUSSIONS

Improving the quality of the skin is neither a simple nor an easy activity. The complexity of this process lies in the way of expression and the diversity of the characters that participate in obtaining quality skins. In view of the fact that many of them are subject to subjective assessments, a situation in which the selection is dependent on the skill and the way a property is perceived by the breeder, the process of skin improvement can be quite long and with very varied results, such as meaning and mode of expression.
This effect is a consequence of the fact that the promotion between the selection criteria of a certain character often determines a simultaneous answer and for another character with which it is in direct correlation. For example, van Niekerk (quoted by Taftă et al., 1997) demonstrate that the improvement of the skins for the uniformity of the curl and the arrangement of the curls on the skin surface, but also of the predominant pattern, entails not only a reduction in the length of the fibers but also an increase in the number of curls with reduced height. However, the effect is totally different when the selection insists on promoting the characters on which the quality of the coating fibers as a whole depends.
By evaluating the characters that were the subject of research, the aim was to find out what is the real effect of the applied selection and what are the progresses made in improving the quality of the skins in the Karakul of Botoșani breed. Following the data processing, the proportion of individuals that have been identified as having good strength and elasticity is recorded to be inconsistent (Table 1).
Analyzing the data resulting from centralization and statistical processing, we find differences between the average values resulting from the assessment of the resistance and elasticity of the curl to the color varieties found in the Karakul of Botoșani breed.

The highest average value was obtained from the evaluation performed on lambs belonging to the black variety. In this case the average score was 45.19 ± 0.45, being less than five points compared to the maximum accepted level for these characters, the maximum accepted score being 50.

The analysis of the evolution of the proportion of lambs that received a high average score highlights the fact that the improvement is certain, being supported by the genetic progress currently registered. Thus, if in 2005 the proportion of lambs that had a good expression for strength and elasticity was 68.12%, their share increased to over 80% in 2020 indicating an improvement for these characters in progress but also the fact that under the effect of selection and the pairing management in each generation increases by approximately 1.25% the number of those who obtained a maximum score in the evaluation.

Table 1. Statistical parameters for the resistance and elasticity of the curl (time to return to the original form)

Color variety	n	$\overline{X} \pm s_{\bar{x}}$	V%	% with a desired strength and elasticity				The difference ± 2005/2020	
				2005	2018	2019	2020	Total (points)	Generation (%)
Black	1501	45.19±0.45	21.83	68.12	80.55	84.56	80.63	12.51	1.251
Grayish	1181	42.73±0.33	26.58	57.36	61.59	72.08	69.47	12.11	1.211
Brown	428	41.24±0.57	28.95	60.88	75.57	73.05	70.25	9.37	0.937
Grey	103	37.86±1.23	33.16	57.13	67.46	64.76	64.29	7.14	0.714
Pink	530	39.65±0.53	31.14	38.19	40.73	45.94	46.12	7.93	0.793

Table 2. The difference and significance of difference for the resistance and elasticity of the curl

Character 1	Character 2	Mean difference	Signification of difference	Level of signification
Gray	Grayish	3.08	insignificant	-
Gray	Brown	1.59	insignificant	-
Gray	Black	1.79	insignificant	-
Gray	Pink	4.87	significant	0.01
Pink	Grayish	3.37	significant	0.05
Pink	Brown	7.32	significant	0.01
Pink	Black	2.45	insignificant	-
Black	Brown	1.50	insignificant	-
Brown	Grayish	3.08	insignificant	-
Black	Grayish	2.46	insignificant	

When evaluating the average score obtained by the lambs of the five color varieties that were included in the experimental protocol, there are differences in the mean values (Figure 1).

In lambs belonging to the grayish color variety, the tendency and evolution of the improvement of the degree of resistance and elasticity follow, in general, the same tendency as in black lambs. The difference is that the average value of the score is only 42.73 ± 0.33 points, being 2.46 points lower than that obtained for black lambs. However, the difference is not significant for P<0.01 (Table 2).

Also, the fact that the proportion of lambs that were evaluated with the maximum points was 57.36% in the 2005 generation and reached only 69.47% in 2015 shows that the improvement of the strength and elasticity of the curl is slower at grayish variety (Figure 2).

However, the selection applied shows that in each generation the proportion represented by individuals who obtained the maximum score in the evaluation of these characters' increases by 1.2%.

However, we can say that in the variety of grayish the improvement process is slow. The relatively slow pace of the genetic improvement process is largely due to the differences found between the traits on which the quality of black and white colored fibers depends. Usually the fibers are longer and are associated with a lower elasticity and strength and the black fibers being shorter are associated with a better expression of that character.

Therefore, it can be specified that when it will be possible to promote through the applied selection only breeders that have fibers in the curl with close length and thickness, and the chances that the improvement will register higher levels will increase.
Between grayish and the pink and black varieties, there are significant differences for P≤1 and insignificant for gray and brown (Table 2).

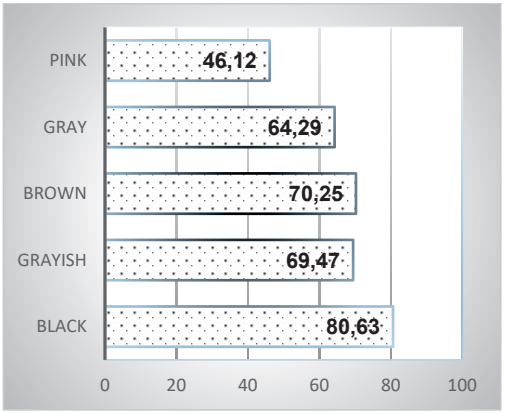

Figure 1. Graphic representation of the average values of the score obtained when evaluating the characters in the lambs obtained in 2020

In order to obtain a curl with more valuable characters, it is necessary to put a greater emphasis in the future selection on the improvement of the dermal layers as they represent the main organ in which the process of morphogenesis of the fibers that constitute the curl takes place. The quality of the skin is extremely important because in the dermal layers the processes specific to the morphogenesis of wool fibers are initiated and carried out. Many studies and research highlight the role and importance of dermal layers, and Karakul has shown that the type of curl, the size of the curl and the quality of the fibers in the curl largely depend on the quality of the skin (Nel, 1966; Schoeman, 1998; Nel, 1969; Van Niekerk et al., 1972; Gouws, 1974).
A larger study conducted in the African Karakul concludes that the expression of some characters followed in the amelioration process is directly influenced by the thickness of the skin and the quality of the covering fibers (Thompson, 1938; Ursu et al., 1997; Wahl et al., 1920; Schoeman, 1998).
In the brown variety, the average score of 41.24 ± 0.577 shows a very good proportion of those who had a good degree of evaluation for the strength and elasticity of the curls. The fact that the proportion of lambs with the desired type of character increases in each generation by about 9.37% shows a good efficiency of selection and an obvious genetic progress, but also a higher degree of improvement.

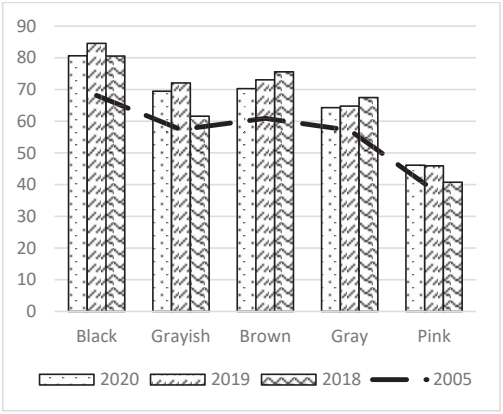

Figure 2. Frequency of character represented by resistance and elasticity (%)

The difference between the average score obtained for brown with brown, brown with gray and brown with black is insignificant for P<0.01.
In the grey variety the improvement of this character is at a lower level compared to the authorized varieties. The mean score of 37.86 ± 1.237 is 24.28% lower than the maximum permitted by the quality certification instructions for purebred animals.
The determination of a progressive increase of the lambs that received the maximum points in the evaluation of these characters indicates that the improvement is on favorable coordinates, even if the genetic progress was identified in only 0.7% of the individuals obtained from the herd of females registered in the breed register.
The pink variety, although obtained a better average score (39.65 ± 0.536), the fact that, so far, the proportion of lambs with a maximum score is below 50% shows a difficult improvement for these characters.

The slow rate of improvement is due primarily to the small size of the active population, with effects on the intensity of selection and, secondly, to the fact that red and white fibers with different lengths and thicknesses are found in the curl structure, influencing the expression of these traits in the genotype.
Determining the degree of significance between the average scores for the grey and pink varieties are significant for P<0.01 and still significant for P<0.05 between pink and grayish.

CONCLUSIONS

The researches values and also the evaluation of the degree of improvement for the specific characters of the skins is supported by the fact that the biological material was represented by the lambs from the sheep registered in the Genealogical Register (Principal and Secondary Section) representing, in fact, the most valuable nucleus of Karakul of Botoșani sheep breed.
Assessing the degree of improvement for the strength and elasticity of the curl in lambs that belong to the black variety, the improvement of this character is in a more advanced process because the average score is about 91% of the maximum value that can be attributed to evaluation.
The fact that the proportion of lambs that received the maximum score in 2005 was only 57.36% and reached as of 2020 only 69.47% shows that the improvement of the strength and elasticity of the curl is slower in the grayish variety.
Between the average scores for resistance and the elasticity of the fibers from grayish with pink, but also from grayish with black, there are significant differences for P<0.01 and insignificant between grayish with gray and brown.
In the brown variety, the average score obtained when evaluating the strength and elasticity of the curs was 41.24 points, which indicates that a very good proportion of individuals found the desired shape.
In the grey variety, the improvement of the resistance of the curl is at a lower level (reduced by 24.28%) than the maximum accepted by the instructions for certification of the quality of biological material in purebred animals.
In the pink variety, the average score obtained was 39.65 points and the fact that, so far, the proportion of lambs with a maximum score is below 50% shows a difficult improvement for these characters.
The slow pace of improvement of these characters in pink lambs is due primarily to the small size of the active population, with effects on the intensity of selection and, secondly, to the fact that in the curl structure are found brown and white fibers that have a length and a different thickness, influencing the expression of these qualities in the genotype.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

OBSERVATION ON LACTIC ACID MODIFICATIONS, DEPENDING ON TRAINING PERIODS AND SPEEDS, IN SPORT HORSES, USED AT EFFORT TEST

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Abstract

In the actual stage, on international level and in our country, is growing the importance of horses for equestrian sports. The researches were carried out on sports horses from Romanian Sport Horse and English Thoroughbred breeds. In this study, in order to evaluate aerobe energetic capacity of the horses, by determining the value of V_{La4}, there was used a standard effort test. There was completed an instrumental method to determine the lactic acid, based on enzymatic reactions and Screen Master Plus spectrophotometer was used for analyses. In can be observed that only for heating stage of training, the average value of lactic acid increases from first test to the third test. At all other effort stages the situation is opposed. Testing the differences significations for the first two testing periods, it was find that between all compared values the differences are very significant. For the third testing period, for all compared lactic acid values the differences are significant, excepting the differences testing between the lactic acid values at 400 m/minute stage and 450 m/minute stage, where the differences are very significant.

Key words*: effort test, lactic acid, sport horse.*

INTRODUCTION

At the present stage at worldwide level and in our country, the importance of the horse for equestrian sports is increasing. As a result, horse breeding technology must also adapt to these requirements and an important place must be given to sport horse dressage and specific training. Horse training mean the physical and psychological preparation of the horse for trials (tests), so that the animal can show at some point all the power it is capable of, without harming its health. Training activities try to reduce the fat layer, to improve the functionality of internal organs, to get muscles to be used to strong and continuous tensions.

When it comes to assessing the physiological adaptations that occur within the muscles of a horse due to training, several approaches are possible: 1. follow-up of the evolution of performance parameters, such as speed, heart rate and blood lactic acid, throughout consecutive standardized exercise tests on a, for example, two-weekly basis, 2. follow-up of muscle morphometric by means of ultrasound (Van de Winkel et al., 2016) 3. longitudinal follow-up of specific parameters, such as muscle fiber typing, total glycogen content, enzymatic activity, in serially harvested muscle biopsies (Vermeulen et al., 2017).

Trainers are familiar with the appearance of lactic acid following muscle effort, even if there are different opinions about the effect it has. Lactic acid is produced in muscles as a consequence of the generating of anaerobic energy and can be metabolized in muscles where it accumulates during critical periods with maximum effort (Dunnett, 2016.)

Lactic acid is an indicator of the effort made by horses during training and it has nothing to do with fatigue or muscle damage and is not harmful. Lactate is a source of energy and helps reduce the acidity of muscle cells subjected to intense training by eliminating hydrogen ions. High levels of lactic acid in the blood can be correlated with high athletic performance, not fatigue.

After training, horses become accustomed to the type of effort required and have normal metabolic levels of acidosis and lactatemia, depending on the effort required they have undergone. However, one hour after effort, only

acidemia was eliminated, while hyperlactatemia was still present (Gomes et al., 2020)

There are several training methods, to prepare the horse for speed trials, endurance or strength tests. Training is like dressage an art, because it addresses to an animal that has a phenotypic appearance and an energetic possible temperament that varies from one individual to another, so that the training methods must be adapted to each individual in a special way.

The sport horse is submit to great efforts and as a result there are made progressive morpho-functional changes of the body, based on a rational, planned and systematic training program. In the process of formation and development of the energetic capacity, based on some schemes and judicious progressions is ensured the maximum improvement of the physiological functions.

At the basis of sport horse optimal preparation must be the following specific principles (Nicolescu, 1950).

a) The principle of sports training. It involves the repeated effort demand of the muscular system, which determines the improvement of the superior nervous activity, the elaboration and establishment of the necessary conditional connections, of the motor qualities and skills the morpho-functional improvement of all the organs and systems of sport horses.

b) The principle of systematic character. In horse training there must be a cadence. The interruption leads to the decrease of working capacity, as a result of the abolition of the previously elaborated and unmaintained connections. There may also be a regression of motor skills, due to the involution of all morpho-functional progressive changes in the body: muscles, cardiovascular and respiratory system, which reduce their activity after decreasing of formed reflexes. If arrhythmias occur in the preparation of the horse, it is first recommended to restore the coordination capacity (easy exercises in manege) and then to do more intense exercises to increase the functional level of the body.

c) The principle of assuring the optimal effort interval and the alternation of effort moments with relaxation ones. During the training period (daily, weekly, monthly, seasonal, annually) the effort periods must alternate with relaxation periods, in order to ensure good training and normal maintenance and health.

The animal's body goes through 3 phases in the training program: fatigue (reduced effort capacity), restoring the work capacity to the initial level and increasing the effort capacity. Each level has different durations, depending on the intensity and size of the effort and the grade of horse training. The alternation of work with breaks depends on the purpose of the training program and the horse preparation level.

d) The principle of progressive tasks increasing. This principle aims the widening of the work capacity limits, which is achieved by continuously increasing of functional body state, allowing the practice of more difficult movements and increasing training tasks. The progressive increase effort must be foreseen in the training program in all its stages and especially in the horses that are preparing for very heavy trials.

e) The principle of maximum effort capacity. Normally, the horse can perform maximum tasks at the end of the training period, when a series of morpho-functional changes occur that allow to increase the intensity of the effort. (Nicolescu T. 1950). But there are certain situations, in the case of horses prepared for heavy trials (complete riding trial), when during the training period maximum effort tests are introduced (rapid ascent of the slopes), which determines a demand of the body up to maximum limits.

f) The principle of multilateralism, which has as fundamental objective the complete and complex development of the horse. Based on this principle, the systems and devices required during training are developed and the superior nervous activity is perfected. For this purpose, the motor qualities of the animal are developed: speed, strength and breathing, based on various types of training.

MATERIALS AND METHODS

Using horses for sport requires preparation and optimization of physical and mental qualities, both contributing to achieving the desired performance.

Energy metabolism in sport horses is strongly influenced by the intensity and duration of exercise. Thus, in short duration and high

intensity efforts, most of the chemical energy needed for muscle contraction is supplied by lactic anaerobic metabolism. This metabolic pathway lead to lactic acid byproduct, whose accumulation, in muscle and blood, will influence the level of serum electrolytes (Şovărel et al., 2014)

The research was carried out on a number of 15 sport horses from the Romanian Sport Horse and Pure English Blood breeds.

To evaluate the aerobic energy capacity by establishing the value of V_{La4} (the horse speed at which the blood lactate concentration reaches 4 mmol and appear the anaerobic metabolic threshold "artificially determined"), a standard test exercise was used, adapted to the specific conditions of the training ground, for three different workouts periods.

The V_{La4} parameter is considered as a reference parameter in horses because it is related to aerobic capacity (Harkins et al., 1993; Persson & Lydin, 1983).

The importance of establishing V_{La4} lies in the fact that it is a method of accurate assessment of the degree of physical training in the sport horse, offering the possibility to constantly check the improvement of aerobic exercise capacity.

The test was repeated in 3 different periods (1st, 2nd and 3rd), during which the horses were gradually trained. This test-exercise is detailed in the Table 1, and can be performed both on the treadmill and on the field.

Table 1. Standardized test-exercise for the determination of $_{VLa4}$ (adapted from Lindner, 1997)

No.	Parameter	Specification
1	Heating	5 minutes to trot
2	Effort level	4
3	Duration of each level	5 minutes
4	Speed in the first level	350 m/minute
5	Increase speed from level to level	With 50 m/minute
6	Rest between levels	1 minute
7	The moment for collecting blood samples	After heating and as soon as possible after each level
8	Recovery phase	10 minutes (step, trot)
9	Sample processing	On the spot and within 30 minutes of sampling

In order to maintain a constant speed during the effort levels, the distance of the course was measured and marked with flags, and each rider, by successive tentative, was framed in the time necessary to cover it. These tentative took place 3 days in advance of the date of the test-exercise. In order to minimize any errors related to the horse-rider relationship, each horse was ridden by the person who prepared it until the time of the test, and the field of exercise was the one used daily for training. The blood sample were harvested from jugular vein. To minimize the stress associated with taking blood samples, the jugular vein was catheterized, using a catheter 18 G/L, 32 mm, which was attached to the skin using adhesive tape.

The blood was collected in Li-heparin vacutainers. The plasma was immediately separated by centrifugation and placing it in separate tubes, immersed in water and crushed ice in an isothermal bag at 4°C. Plasma lactate dosing was performed on site within a maximum of 30 minutes of sampling.

The protocol recommended by Lindner (1997) for plasma lactate dosing provides this immediate separation of erythrocytes, as glycolysis continues in blood samples. If the samples cannot be processed on the spot, it is recommended to take the blood on sodium fluoride and store it at 4°C until dosing. Even under these conditions, the lactate should be dosed within a maximum of 48 hours. In order to determine the concentration of lactic acid, there was selected a specific, instrumental method, based on enzymatic reactions. The principle of the method consists in the transformation of lactic acid into pyruvic acid under the action of lactat-oxidase (LOD), a process in which rises an equivalent amount of hydrogen peroxide. The hydrogen peroxide formed is coupled with 4-aminoantipyrine (in the presence of peroxidases), forming a coloured compound. The intensity of the colour is proportional to the concentration of lactic acid.

Blood samples were collected in vacutainer tubes, on lithium-heparin with the addition of fluoride. The most difficult part of this analysis is stabilizing the level of lactic acid to the corresponding concentration level to that in vivo. The in vitro increase of lactate concentration is prevented by reducing glycolytic activity, which is why the blood is collected on sodium fluoride (or it is deproteinized and the precipitate is separated immediately by centrifugation). After sampling, lactic acid was

immediately measured from the separated plasma. For the analyses it was used the Screen Master Plus spectrophotometer.

RESULTS AND DISCUSSIONS

Regardless of the test period in which the investigations were made, the lactic acid content was the lowest in the heating phase and increased in relation with the speed of movement.

The lactic acid values determined in different phases of the exercise test (from heating to increasing speeds from 350 m/minute to 500 m/minute), determined in 3 different periods, are presented in Table 2.

Table 2. Average lactic acid values on the exercise test

Testing period	n	Lactic acid values ($\bar{x} \pm s_{\bar{x}}$ mmol/l)				
		Heating	350 m/min	400 m/min	450 m/min	500 m/min
1	15	0.46 ± 0.019	1.86 ± 0.032	3.84 ± 0.07	5.08 ± 0.12	10.61 ± 0.39
2		0.60 ± 0.04	1.83 ± 0.06	2.61 ± 0.05	4.05 ± 0.08	7.74 ± 0.17
3		0.65 ± 0.03	1.63 ± 0.46	2.44 ± 0.05	3.62 ± 0.10	4.12 ± 0.03

It is observed that only at the heating phase the average value of lactic acid increases from the first test to the third test. At all other levels of effort the situation is reversed, the value of lactic acid decreasing from the first test to the third.

It is observed that in the first testing period, at a speed of 400 m / min., the value of the lactic acid concentration it is close to V_{La4}, that is 3.84 mmol/l, which means that the training intensity approaches the optimal level.

In the same test period, at higher speeds (450 m/min. and 500 m/min. the value of V_{La4} was exceed, obtaining 5.08 mmol/l and 10.61 mmol/l, respectively, which may show that the training intensity is not appropriate (figure 1).

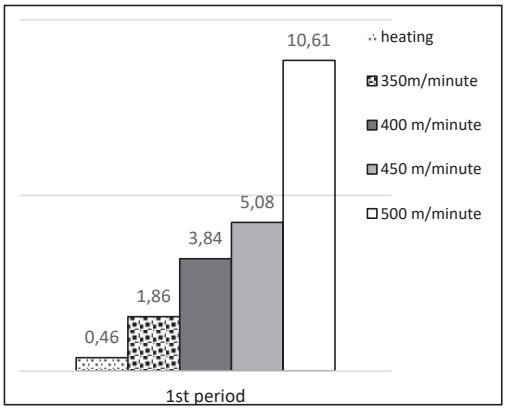

Figure 1. Average lactic acid values at first period of the exercise test

It is observed that in the second test period, the optimal value of the lactic acid concentration (V_{La4}) was not reached at heating and speeds of 350 m/minute and 400 m/minute, but at a speed of 450 m/minute it is optimal (4.05 mmol/l). (figure 2).

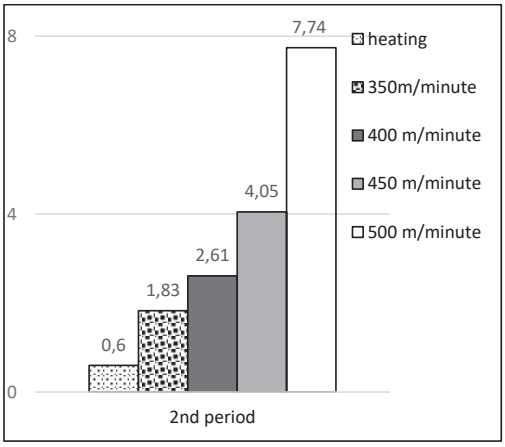

Figure 2. Average lactic acid values at second period of the exercise test

The optimal value of V_{La4} was exceeded, at a speed of 500 m/minute, approaching a double of it (7.74 mmol/l). This may indicate an incorrect intensity of effort and an inadequate training structure

In the third test period it is observed that the value of the lactic concentration was not reached up to the speed of 450 m/minute. At a speed of 450 m/min, the value obtained is close to V_{La4}, and at a speed of 500 m/minute it can be said that it reached the optimum value (figure 3).

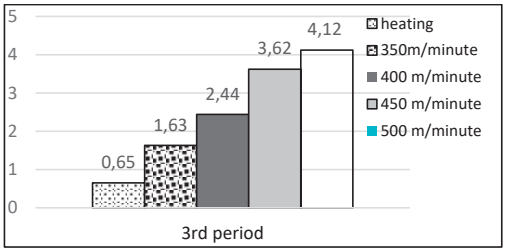

Figure 3. Average lactic acid values in the third period of the exercise test

Testing the significance of the differences for the first and the second test periods, it was find that between the value from heating and the value achieved at a speed of 350 m/minute the differences are very significant (Tables 3 and 4). It can also be seen that between the values compared at speeds of 350 and 400 m/minute, 400 and 450 m/minute and 450 and 500 m/minute, the differences remain very significant (Tables 3 and 4).

Testing the significance of the differences for the third test period, we find that between all the compared values, the differences are significant, except for testing the differences between the values from the 400 m/minute and the 450 m/minute level, where the differences are very significant (table 5).

Table 3. Significance of differences for the first test period

Specification	I					
	$\overline{X}_1$	$\overline{X}_2$	d	t calculated	table t (t α) P<0.001	Signification
Heating-350 m/min	0.46	1.86	-1.4	**-12.2**	**3.67**	Very significant differences ***
350 -400 m/min	1.86	3.84	-1.98	**-12.2**		Very significant differences ***
400 -450 m/min	3.84	5.08	-1.24	**-5.59**		Very significant differences ***
450 -500 m/min	5.08	10.61	-5.53	**-15.23**		Very significant differences ***

Table 4. Significance of differences for the second test period

Specification	II					
	$\overline{X}_1$	$\overline{X}_2$	d	t calculated	table t (t α) P<0.001	Signification
Heating-350 m/min	0.60	1.83	-1.23	**-7.65**	**3.67**	Very significant differences ***
350 -400 m/min	1.83	2.61	-0.78	**-4.63**		Very significant differences ***
400 -450 m/min	2.61	4.05	-1.44	**-7.86**		Very significant differences ***
450 -500 m/min	4.05	7.74	-3.69	**-15.48**		Very significant differences ***

Table 5. Significance of differences for the third test period

Specification	III						
	$\overline{X}_1$	$\overline{X}_2$	d	t calculated	table t (t α) P<0.01	table t (t α) P<0.001	Signification
Heating-350 m/min	0.65	1.63	-.098	**-2.755**	**2.756**	-	Significant differences **
350 -400 m/min	1.63	2.44	-0.81	**-2.23**			Significant differences **
400 -450 m/min	2.44	3.62	-1.18	**-5.99**	-	**3.67**	Very significant differences ***
450 -500 m/min	3.62	4.12	-0.5	**-2.73**	**2.756**	-	Significant differences **

CONCLUSIONS

Adaptation to effort for sport horse with performances, involves physiological adjustments in the striated muscles, respiratory system, cardiovascular system, endocrine system and all organs ad body systems. In order to cope and reach the optimal and maximum potential, the animal's body subjected to high efforts, is necessary to carry out a training with appropriate intensity, which will lead to certain biochemical changes in the blood.

As a result of the training performed, the blood lactic acid values changed, being around the critical value at a speed of 400 m/minute (V_{La4} = 3.84 ± 0.07 mmol/l), in the first test period, reaching the optimum value at a speed of 450 m/minute (4.05 ± 0.08 mmol/l), in the second test period and slightly exceeding this value at a speed of 500 m/minute (4.12 ± 0.013 mmol/l), in the third test period.

The maintaining of the training program results in the obvious improvement of the aerobic energy capacities.

In order to maintain the health of the animals and to improve the sports performances, it is necessary to carry out periodic analyses of the biochemical components in the horses and of the main physiological indices, both before and after the effort, in order to notice the situations that would alter the state of health of the animals subjected to too much effort, thus giving the possibility to establish a certain training regime. It is also necessary to establish an optimal training regime (daily and monthly), with the progressive increase of the effort to which the animals are subjected (increasing the speed of movement in the test-exercise).

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

STUDY OF THE MAIN BODY DIMENSIONS THAT ARE USED IN THE SELECTION PROCESS, IN THE REPRODUCTIVE NUCLEUS OF THE ROMANIAN TROTTER HORSE FROM DOR MARUNT STUDFARM

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Abstract

This study is a part of an ample research regarding racing performance's improvement of Romanian Trotter horse breed. Study of average values of the main body dimensions that are used in horse selection process can offer an idea about the population genetic level, because, regarding to a population, the average of phenotypic value is equal with average of genotypic value. The main body dimensions that was analysed in this study and who are used in the selection process of horses in Romania, is: withers height, thoracyc perimether and cannon bone perimether. All this evaluation of Romania Trotter horses through the integration of individs in an evaluation class, it was made in accordance with selection methodology. The biological material is represented by 62 Romanian Trotter horses, 8 males and 54 females, at different ages, owned by Dor Marunt studfarm. The average performances of characters are presented in the paper. We can observe a small grade of variability with some differences between sexes. The average performances of the characters are between characteristic limits of the breed.

Key words*: horse, racing, Romania, selection, trotter.*

INTRODUCTION

Regarding to a population, the average of phenotypic value is equal with average of genotypic value. So, the studies of the average value of characters offer us an idea about the population genetic level (Marginean, 1997). This study have the principal purpose to analyse main body dimensions, of Romanian Trotter horse, who are used in selection process: withers height, thoracyc perimether and cannon bone perimether, through the integration of individs in an evaluation class, in accordance with selection methodology (Marginean et al., 2005; Maftei et al., 2015).

MATERIALS AND METHODS

The biological material is represented by 62 Romanian Trotter, 8 males and 54 females, at different ages, owned by Dor Marunt stood farm, representing the entire reproductive nucleus from the last years. The individuals were analyzed through individual performances, through stallions average performance, through mares average performance, and, most important, through population average performance of body dimensions, related to selection criteria (Popa, 2009). We analyzed the withers height, the thoracic perimeter and the cannon bone perimeter using body measurements. All measurements were made in Dor Marunt studfarm, using an ANIMETER measuring belt and a height measuring stick. The males values are presented in Table 1.

Table 1. The body measurements values for stallions

No. crt.	Name	Withers height	Thoracyc perimether	Cannon bone perimether	Obs.
1	CHIRONE DEI	153	180	20.5	Imported
2	SELF OBSESED	156	182	20	Imported
3	NUROFEN	159	177	19	Romanian Trotter
4	VIS	151	182	19	Romanian Trotter
5	BIZAR	156	182	19	Romanian Trotter
6	OLIMP	157	184	20	Romanian Trotter
7	VARTEJ	153	180	19	Romanian Trotter
8	NELUTU	158	180	19	Romanian Trotter

For mares, the measurements for all three dimensions that was take in to the study, is presented in Table 2.

Table 2. The body measurements values in mares

No. crt.	Name	Withers height	Thoracyc perimether	Cannon bone perimether
1	KINTA	157	184	19
2	RECEPTIA	157	175	19.5
3	CAMILA	154	173	20.5
4	STEMATA	155	171	19
5	VRAJA ZORILOR	154	174	19
6	SIAMEZA	156	177	19
7	BRENDA	156	182	19
8	KATRINA	156	178	19
9	RAZA DE LUNA	157	182	20
10	SIMETRIA	152	178	19
11	SOGUNA	157	178	20
12	IALTA NU	158	182	21
13	SOLOMIA	155	184	19
14	NEVADA	154	180	20
15	VRAJA SOU	153	184	19
16	REGINA ANA	156	184	19
17	KATIUSA	154	178	19
18	ROMANITA	157	172	19
19	DIACONITA	158	180	20
20	OSANDA	157	184	19
21	PAMFILA	154	178	19
22	VRAJA LIREI	156	178	19
23	AMICA III	154	182	19
24	PAMELA	153	184	19.5
25	NEDORA	154	180	19
26	ONDA	156	180	18.5
27	SARA	154	184	18.5
28	VOIAJORA	153	178	18.5
29	RELAXA	158	184	19
30	REGINA ANTOANETA	155	182	19.5
31	SULTANA	155	180	19
32	VICTORIA	152	180	19.5
33	KITTY	156	184	19
34	SORANA	151	180	19
35	KISS ME	158	184	19.5
36	SERENA	161	180	19
37	IRENA	159	180	19.5
38	FINUTA	158	184	19
39	VRAJA STANCA	154	186	19
40	SENIORITA	155	180	19
41	PATIMA	156	182	19
42	RASFATATA	158	184	19.5
43	VENERA	154	182	19.5
44	PANDORA	154	178	18
45	OPS	157	178	18
46	ASTARTE	158	184	19
47	VIDIA ROSIE	156	175	18
48	VRAJA ZAPEZII	155	178	18
49	KIRRA	161	182	19.5
50	RAMYA	156	179	18.5
51	NEMARA	155	179	18.5
52	KINA	154	176	18.5
53	SOLEDAD	159	176	19
54	SOPHIA	158	182	18.5

RESULTS AND DISCUSSIONS

Analyzing recorded data, we can observe some unsignificant differences, for all characters, between stallions (Figure 1), between mares (Figure 2) and at population level (Table 3, Figure 3).

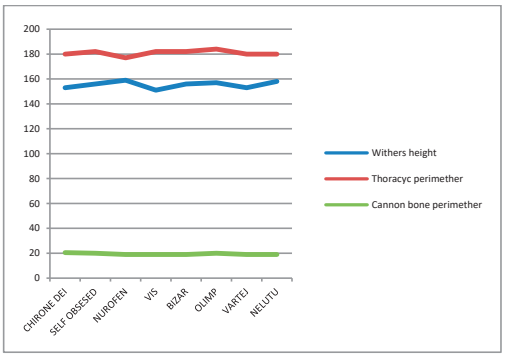

Figure 1. Wither's height distribution of values in stallions

In stallion case we find unsignificant differences between individual performances, evan if we analyzed six Romanian Trotter stallions and two imported stallions. The bigger value of withers height is 159 cm (stallion Nurofen, a Romanian Trotter), and the smallest was 151 cm (Vis stallion - Romanian Trotter). The other two imported stallions cannot influence the average value of character: stallion Chirone Dei - 153 cm, and Self Obsessed - 156 cm. The average performance of stallions was 155.38 cm, corresponding not to the best class (Record) and only to the next one (note 8 - Elite class). We must specify that the improving of wither's height values in Romanian Trotter it is a concern of specialists from this domain.

The thoracic perimeter value, was maximum 184 cm (Olimp - Romanian trotter stallion), and minimum 177 cm (Nurofen, Romanian trotter stallion). Average performance for thoracic perimeter was 180.88 cm, corresponding for note 10, respectively record class. Canon bones perimeter have maximal value at 20.5 cm (imported stallion Chirone Dei), and a minimal value at 19 cm (Nurofen, Vis, Bizar, Vartej, Nelutu), with an average value of 19.44 cm. Average value corresponding for note 9 but also for record class. Overall, regarding

corporal dimensions, the stallions meet criteria's to be sires.

In the mares effective, we cannot say that we have a similar situation. For withers height, we record a maximal value at 161 cm (Kira and Serena), and a minimal value at 151 cm (Sorana). The average performance was 155.74 cm, corresponding for note 8, respectively elite class.

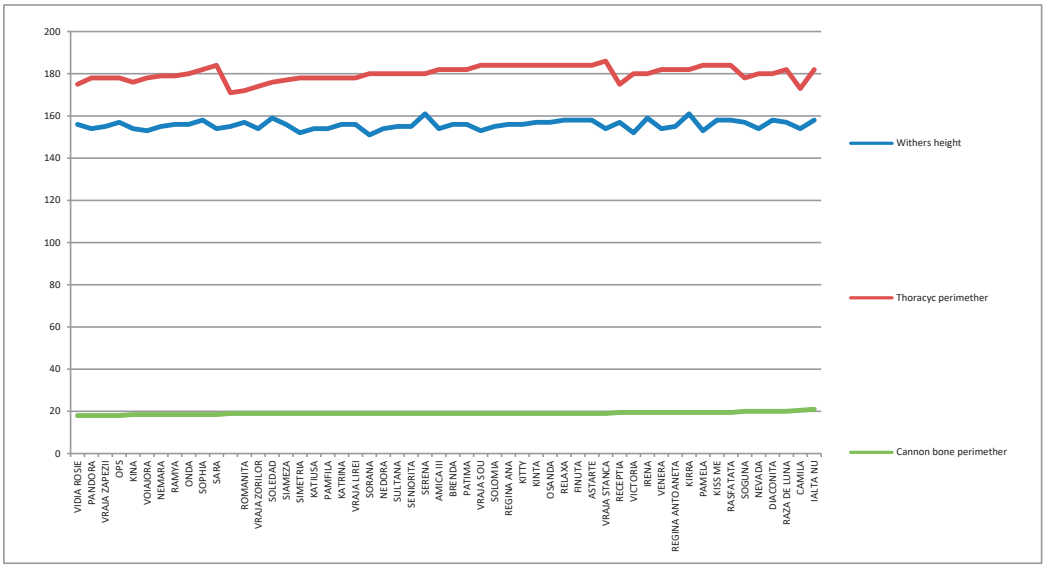

Figure 2. Wither's height distribution of values in mares

Table 3. Average values of analyzed characters (in cm) and statistics

Specifications		Wither's height	Thoracyc perimether	Cannon bone perymether
Males	***X***	155.3750	180.8750	19.4375
	STDEV	2.7742	2.1002	0.6232
	Sx	1.0486	0.7938	0.2356
	cv%	1.7855	1.1611	3.2062
Females	***X***	155.7407	180.0556	19.0833
	STDEV	2.1385	3.4827	0.5807
	Sx	0.2937	0.4784	0.0798
	cv%	1.3731	1.9342	3.0432
TOTAL	***X***	155.6935	180.1613	19.1290
	STDEV	2.2072	3.3348	0.5932
	Sx	0.2826	0.4270	0.0760
	cv%	1.4177	1.8510	3.1012

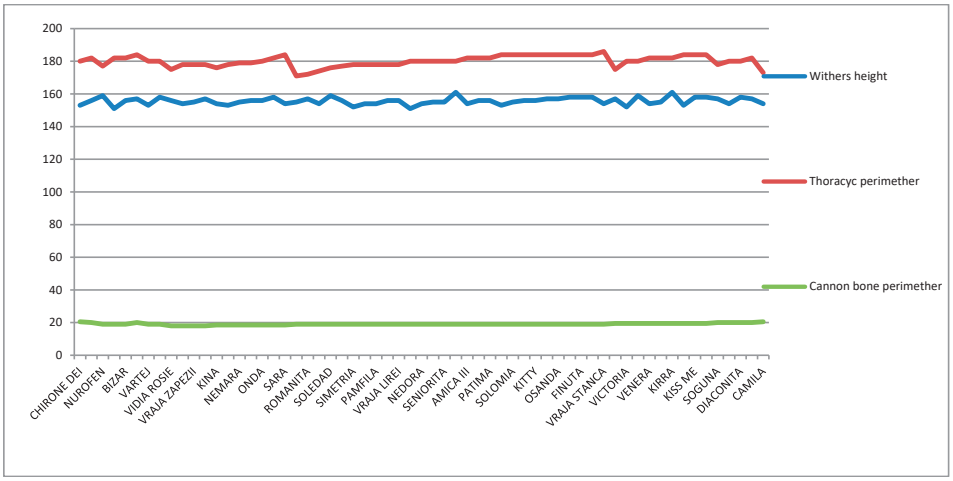

Figure 3. Distribution of character values in analyzed population: Romanian Trotter from Dor Marunt studfarm

Table 4. Average values of analyzed characters (in cm), and statistics

Stallions			Note	Mares		
Wither's height	Thoracic perimeter	Cannon bone perimeter		Wither's height	Thoracic perimeter	Cannon bone perimeter
164	184	20.5	9	164	186	20
162	182	20	10	162	184	19.5
160	180	20	10	160	182	19.5
158	178	19.5	9	158	180	19
156	176	19	8	156	178	18.5
154	172	18.5	7	154	175	18.5
152	168	18	6	152	172	18
150	166	17.5	5	150	168	17.5
-	-	-	4	148	166	17.5

Table 5. Ranking of Romanian Trotter by body dimensions

Specification	Wither's height		Thoracyc perimeter		Cannon bone perimeter	
	Stallions	Mares	Stallions	Mares	Stallions	Mares
Body dimensions	9	8	8	7	7	7

Figure 4. Aspects from testing process of Romanian Trotter

The thoracic perimeter had values between 186 cm (Vraja Stanca) and 171 cm (Stemata), with an average of 180.06 cm. However, the average performance includes mares stock in record class.

Regarding the cannon bone perimeter, 28 mares was recorded a value of 19 cm and only two mares had recorded over 20 cm for this character.

The average performance was 19.08 cm, corresponding for note 9 - record class.

Figure 5. View from a National studfarm

Regarding the ranking of Romanian Trotter through the average performances on body measurement, separate by sexes, it is obvious that the individuals qualify in class RECORD (Tables 4 and 5).

CONCLUSIONS

Analyzing the reproductive nucleus of Romanian Trotter horse from Dor Marunt studfarm, by average values of corporal dimensions, it's obvious that the individuals deserve to be the ancestors of a new generation. We strongly recommend increasing of reproductive nucleus, for this breed, associated with a very strong control of selection. Also, selection of individuals with wither's height values a little bit bigger could be the answer for low performances of Romanian Trotter in long racing. Do not forget that it is a must to continue by using stallions from import with a good racing performance but also with the exceptionally genetic value, qualities that can make them succeed in reaching the status of Sire Stallions in the reproductive nucleus of Romanian Trotter. However, due to the small values of variability we can say that the average performances of characters is able to offer us a good and correct image of Romanian Trotter horse population from Dor Marunt studfarm.

ACKNOWLEDGEMENTS

This research work was carried out with the support of National Forestry Authority – Horse Breeding Department.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

STUDIES ON BEEF CATTLE FOR PROCESSING IN ROMANIA

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Abstract

In our country in recent years we can see a decrease in cattle from 2330 thousand heads in 2018, to 1914 thousand heads in 2020, a decrease due to, among others, the faulty organization of breeders in order to capitalize on the production obtained and a structure low size of cattle farms. In 2020, the number of cattle slaughtered in industrial units (slaughterhouses) decreased compared to the previous year by 15.6%, beef production decreased from 196,000 tons in 2018 to 170,000 tons in 2020, even if the yield at slaughter has increased. Thus, there is an increase in the average body weight of cattle from 433 kg/head to 450 kg/head, this is due to advanced, cost-effective breeding technologies and optimal animal nutrition. All this influences, of course, the quality of the meat, which will be superior and will have a high yield for processing. Meat consumption in Romania is 7 kg per capita per year, the demand for beef increases significantly in Romania, being a large deficit of high quality animal protein, the Romanian unfortunately preferring pork and chicken.

Key words*: beef cattle, cattle breeding, meat consumption, slaughter yield.*

INTRODUCTION

Beef is a major source of meat for most peoples. The total world demand for animal products is covered by approximately 42% of developed countries, 12% of developing countries in group I and over 46% of those in group II (Nistor-Anton & Maciuc, 2020; Ripoll et al., 2018; www.Fao-org.com).

The most suitable meat production technologies are sought in all countries by minimizing costs, maximizing increase and weight at slaughter.

In the U.E. there is a diversification of fattening technologies: milk veal or white meat, beef and beef. White meat is in demand in France, Italy, Switzerland, Spain (Holtcamp et al., 2019).

There are two types of beef in Romania: imported, which comes frozen from Argentina, Brazil, New Zealand and another produced in Romania, on a smaller scale. The differences are significant both quantitatively and qualitatively. Frozen meat loses its physico-chemical and organoleptic qualities, which a fresh beef has.

In Romania, in this species, meat production is ensured from the reformed adult and semi-adult cattle, but especially from the fattened male youth and a small share from crossbreeds of meat and meat breeds (Maciuc et al., 2016; Liciu, 1999; www.insse.ro).

Beef consumption in Romania amounts to 7 kg/inhabitant, according to reports from processors and slaughterhouses. Worldwide, the average consumption of beef is 12 kg/inhabitant. Therefore, in Romania there is a real need to raise beef cattle. Romania provided 42% of the beef and sheep meat needs for the Middle East before 1990. Today, unfortunately, we import to ensure the needs.

Even if there were imports of specialized breeds of cattle for meat production: Aberdeen Angus, Charolaise, Limousine; Blonde of Aquitaine, Heroford, Galloway; Highland; Aubrac; Bălțată Românească - SIM, etc., the total number of cattle is still small, and Romania from the exporting country has come to import beef (Pesonen et al., 2012; Holtcamp et al., 2019; Jiu et al., 2020).

In the future, beef production will also belong to family farms, but the base will be large production units of industrial type. Regarding the capitalization of meat production, it will be done in the form of preparations, semi-prepared, in an integrated system.

Representing an important source for food with high biological value, but also appreciated as a means of labor, increasing and improving of the cattle subfamily is for mankind a surplus food, providing about 55-57% of the animal protein consumed daily. The set of technical

and organizational measures in cattle exploit operation involves several processes of feeding, growth, reproduction, breeding, which helps to increase the productive potential (Gociman et al., 2019).
Given the above, the sharp decline in livestock and meat production, the malfunctioning of the breeding system, the lack of a strong meat-producing sector - goods and of course the lack of assorted varieties of such beef products, we have proposed to do this research.

MATERIALS AND METHODS

This research is essentially a study on studies aimed at achieving an integrated result on the number of cattle and meat production obtained. Over a period of three years, 2018-2020, we analyzed the number of cattle, size of farms, number of heads slaughtered, live weight, average weight, carcass weight, but also the evolution of beef for processing.
The research method included, documentation, observation, analysis, statistics and analysis in the field of data description.
Once systematized, the data were processed and interpreted by methods specific to such research - arithmetic mean (X), arithmetic mean error (± s) standard deviation (s), coefficient of variability (V%) and significance test p, using the program of statistics, analysis of variance and covariance (S.A.V.C.).
The complexity of the pursued aspects required the use of a diversified working methodology depending on the pursued aspects, using and respecting the investigation methodology recommended by the specialized literature.

RESULTS AND DISCUSSIONS

The research aimed to achieve an updated situation regarding the total number of cattle in Romania in parallel with the total number of cattle for meat.
This is one of the important aspects, especially for farmers who want to orient their zootechnical specificity towards a subsequent meat processing.
Romanian area is 237,500 km^2 wide, consisting of a symmetrical landforms, concentric and varied, with the main features of landforms proportioned as follows: 31% mountains, 36% hills and plateaus, and 33% plains. Large pasture lands, climate, precipitation variation, soil, quality feed are some of the great strengths of Romania to grow such an extensive cattle breed (Gociman et al., 2020).
Romania is the second country from European Union with surface of pasture, the sixth country from European Union as agricultural area and the ninth country from European Union as number of cattle (Gociman et al., 2019).

Table 1. Dynamics of meat and meat production in the period 2018-2020

SPECIFICATION	UM	2018	2019	2020
Total number of cattle	thousand heads	2330	1985	1914
Total live beef production	thousands kg	196	188	170

Analyzing the data collected from the period 2018-2020, we can see how the total number of cattle registered in our country are in a sharp decline, the approximately 3.5 million cattle missing, about 18% of cattle, in an interval of only 3 years, this aspect reveals a decrease due among others to the faulty organization of the breeders in order to capitalize on the obtained production and a low dimensional structure of the cattle farms (www.fao-org.com; Banu et al., 1999; www.insse.ro).
The sharp decline in livestock and meat production, the malfunctioning of the breeding system, the lack of a strong meat-producing sector - goods and of course the lack of assorted varieties of such beef products, are negative aspects that place our country among the last from Europe in this regard.
However, the number of cattle used for meat is of particular interest, as they are increasingly being raised by small farmers, which create a complex technological flow from raising live animals to using beef products sold in stores.
In conclusion, 51,000 head of cattle were slaughtered in November 2020, an increase of 8.5% compared to October 2020, but a decrease of 7.3% compared to November 2019. Meat production obtained in November 2020 from animals slaughtered reached 8,330 tons, compared to 7,583 tons in October 2020 and 9,274 tons in November of the previous year.

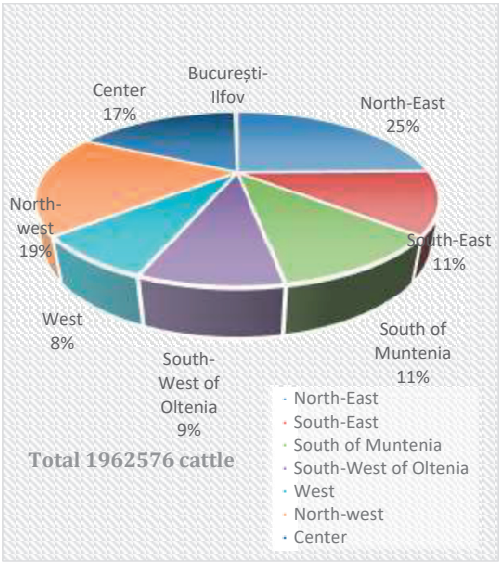

Figure 1. Distribution of cattle in 2019, by development regions according to the National Institute of Statistics

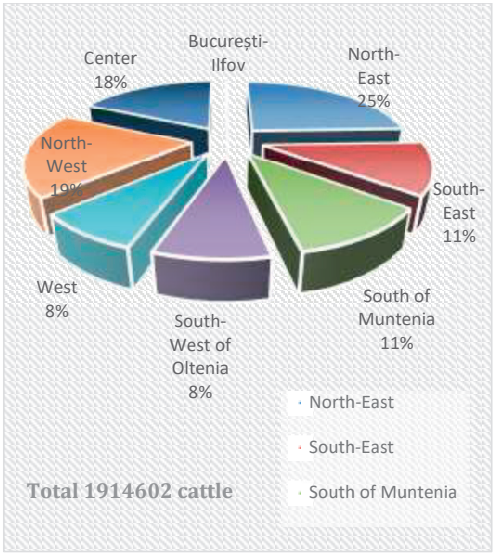

Figure 2. Distribution of cattle in 2020, by development regions according to the National Institute of Statistics

It can be seen that the distribution of cattle in our country has a relatively uniform weight, but nevertheless the Northeast region of Romania is one in which the total number of cattle is higher, a herd of 478,651 cattle. This is largely due to the establishment of a large number of family farms with small livestock.

From the figures presented in parallel on the distribution of cattle by development regions of our country during 2 years, 2019-2020, it can be seen that in the northeastern part of Romania is the largest number of cattle, because climate factors and the forms of relief (hill, plain, mountain) are the most varied in this part of the country. However, the decrease in the total number of cattle can be noticed especially in the southern part of our country. where it seems that the interest in raising animals and practicing these occupations of animal husbandry have been replaced by other activities specific to those warm areas of the plain.

Another trend that is observed in some areas of our country is to raise a relatively small number of cattle for meat 10-50 heads, through cost-effective, economical and beneficial breeding methods for both farmers and consumers.

The table below shows the number of cattle on livestock farms, with the actual number of cattle for each breed, where it can be seen that the number of cattle in the Aberdeen Angus breed is constantly growing in our country.

Table 3. Total number of beef cows included in the 2019 breeding programs

Breed	Number	Holdings
AN	30773	906
CH	4177	206
LIM	2965	136
AU	636	23
SS	391	19
GA	190	7
HI	186	11
TOTAL	39318	1308

According to the data from various information sources, summarized in the tables above, you can see the distribution of cattle breeds according to the size of our farms in the country and beef cattle are the most exploited, their number being in a continuous ascent especially in small farms with 11-50 heads.

Beef obtained from cattle is considered a complete food, with a high protein content and special organoleptic characteristics, it contains 34.6% dry matter, of which 18.6% protein, 15.2% fat and 0.8 mineral salts, with an energy content of 2268 kcal/kg. (Maciuc, 2017; Terevinto et al., 2019; Pesonen et al., 2012).

The total number of heads slaughtered in 2019, according to the FAO was 196037, and the carcass weight was 43536 tons.

Table 4. Size of farms participating in the development of approved breeding programs – cattle

Size of farms (heads)	ABERDEEN ANGUS		CHAROLAISE		LIMOUSINE		AU, HI, GA, HG	
	Number	%	Number	%	Number	%	Number	%
1-10	267	29.5	102	**50**	53	39	18	31
11 – 50	482	**53.3**	87	42	70	**51**	37	**63**
51 – 200	140	15.4	15	7	12	9	3	5
201 – 500	15	1.6	2	1	-	-	1	2
Peste 500	2	0.2	-	-	1	1	-	-
TOTAL	**906**		**206**		**136**		**59**	

Table 5. Slaughter of cattle in industrial units (slaughterhouses)

Species name	Number of slaughter of cattle		Live weight (tons)		Average weight (kg)		Carcass weight (tons)	
	2018	2019	2018	2019	2018	2019	2018	2019
Cattle	233142	196037	101041	87373	433,4	445,7	49922	43536

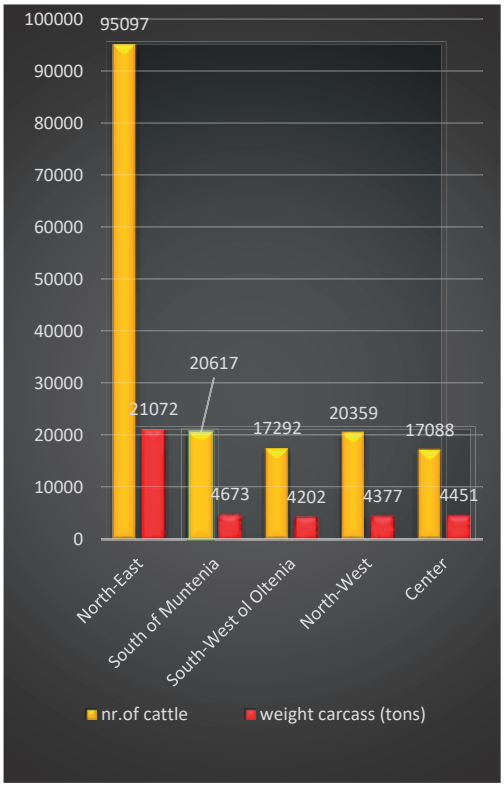

Figure 3. Slaughter of cattle in industrial slaughterhouses

According to INS data, in March 2019, 5,904 tons of meat were obtained from cattle, this production being down by 18.6% compared to March of the previous year (7,265 tons) and by 20.8% compared to February (7,464 tons), the number of animals slaughtered in March 2019 being 37,013 heads. (www.insse.ro).

According to Figure 3, it can be seen that in the northeastern part of the country the slaughter yield and the total number of animals slaughtered for meat has an upward trend, their number is 95097 head of cattle for meat and the carcass weight is 21072 tons, according to data obtained from the Ministry of Agriculture and Rural Development in our country.

This can be an optimistic one, which means that the interest for animal husbandry is growing in this part of Romania, where the breeding farms are developing, and the meat obtained from cattle is capitalized in specific processed products.

In order to increase beef production, the following objectives must be pursued at national level: increasing slaughter weight, which will automatically lead to increased slaughter yields; the adoption of breeding programs to increase meat production, ensuring all the necessary conditions for the animal to externalize its productive potential; boosting the growth of cattle in mountain areas; financial support of breeders (www.madr.ro).

The average carcass weight recorded in November last year was 163.3 kilograms in cattle, INS states that the monthly data on total

animal slaughter are obtained by summing the data from the monthly statistical survey for specialized industrial units (slaughterhouses), which provides data on meat production (number of heads slaughtered, live weight and carcass weight).

CONCLUSIONS

Following the study conducted for the period 2018-2020, the following conclusions emerged:
The total number of cattle is declining due to poor funding programs and insufficiently developed in this species. By increasing the level of production, but also the number of cattle, we can reach the status of exporting country from the group of countries importing meat and meat products.
It was found that farms with between 11 and 50 heads are in continuous development, especially in the North-East of Romania, where the establishment of a number of farms with small numbers is increasing in recent years.
Even though the number of cattle is declining by about 18%, the number of cattle specializing in beef production is increasing. The explanation lies in the fact that the demand for beef is growing and the investments in such a farm are much lower compared to a farm for milk production.
The specialization of the farms determined the increase of the animal's weight at slaughter, implicitly of the slaughter yield, but also of the weight of the quality meat destined for processing.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

THE SUSTAINABLE CONTROL OF VARROOSIS (*VARROA DESTRUCTOR*) BY TREATMENT OF CAPPED HONEYBEE BROOD USING ORGANIC VOLATILE ACIDS AND INNOVATIVE PROCEDURES

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Abstract

The varroa mite infestation is a serious cause of honeybee colony loss at a global level. The varroa mite population development in the honeybee colony is the result of its reproduction success and of some favouring factors. Its parasitism model, which rely on capped brood for reproduction, as well as the role as vector of viruses increase the negative impact on honeybee health. Thus, there is clearly a necessity to develop new treatment approaches to interrupt the mite's life cycle, especially before winter honeybee rearing in order to protect it. Except for the formic acid, the substances used today, which generally treat the whole colony, target only phoretic mites. Using the formic and acetic acids' rapid vaporization properties, two procedures were developed and tested for the treatment of capped brood. The results show a high effectiveness in the mortality of mites (90-100%) in different experimental variants. The capped brood brushing with volatile organic acids represents a highly effective, cost efficient, organic and minimally invasive procedure. It could be applied any time during the active season to decrease the level of infestation before critical moments.

Key words*: brushing, capped brood, honeybee, organic, varroa.*

INTRODUCTION

The worldwide depopulation and mortality of honeybees' colonies in the past decades, caused by different factors, has been widely documented (Potts et al., 2010; Neumann and Carreck, 2010; vanEngelsdorp et al., 2009).

One of the main causes of these mortalities, varroosis, was also largely studied (Traynor et al., 2020; Noël et al., 2020; Nazzi and Le Conte, 2016; Piou et al., 2016; Le Conte et al., 2010), its control being the subject of different, complex strategies (Roth et al., 2020; Dieteman et al., 2012).

Being an important vector for viruses, especially for the deformed wing virus - DWV, (Roberts et al., 2020; Dubois et al., 2020; 2019; Barrosso-Arévalo et al., 2019; Dainat et al., 2012a) and in light of the new findings showing that this parasite feeds primarily on the fat body of honeybees (Ramsey et al., 2018), the negative impact increases substantially, especially on winter honeybees' longevity and immunity (Di Prisco et al., 2016; Annoscia et al., 2015; Francis et al., 2013; Nazzi et al., 2012).

The mite *Varroa destructor* (Acari: Varroidae) (Anderson and Trueman et al., 2000) was described for the first time as the ectoparasite of *Apis cerana*, a species which copes very well with this parasitosis by complex adaptive, naturally selected traits, one of them being the almost exclusive reproduction of the varroa mite in drone brood, (Lin et al., 2018; Beaurepaire et al., 2015; Rath, 1999; Koeniger et al., 1983).

In *Apis mellifera*, varroa mite reproduction takes place in both, drone and worker brood, but there is a preference for drone brood in its rearing period, when the mite population could be 8-10-times greater (Rosenkranz et al., 2010; Boot, 1995; Boot et al., 1995; Boot et al., 1993; Fuchs, 1990). Following the differences in the post-capping period, an average of 1.3 -1.45 new mated females are produced in worker brood and 2.2-2.6 in drone brood (Martin, 1994). The success of its reproduction depends highly on the number of the reproductive cycles per each mated female, with an average of 2-3 reproductive cycles (Donze et al. 1998; Martin & Kemp, 1997; Ruijter et al., 1987), as well as on the type of brood. In the drone brood

it is 95%, while in the worker brood it is 73% (DeGrandi-Hoffman & Curry, 2004).

As it is well known, the life cycle of the varroa mite includes a phoretic phase, visible on adult bees, and a reproductive phase, which takes place in the capped brood, where new generations of mites are reared. Studies show that, in the active season, up to 90% of the varroa mite population can be found within the brood (Rosenkranz et al., 2010). Thus, the reproductive phase of mites has a very important negative impact on honeybees' health as both mature and immature mites feed intensively on brood, affecting the nutritional status and the immunity, as well as transmitting the viruses. As result of this complex varroa-honeybee relationship, combined with seasonal particularities and re-infestation risks, the varroa mite population in a colony is a dynamic process, with different levels of infestation between colonies, regions and time periods (DeGrandi-Hoffman & Curry, 2004; Martin, 1998; Fries, 1994) which trigger the treatment strategies.

Regarding the reproduction phase, the varroa mite foundress enters a cell just before it is capped, for example in a 0-24 hours interval in the case of honeybee worker brood, and an even longer interval in the drone brood (Donze et al., 1998; Ruijter et al., 1987).

In the post capping period, the honeybee metamorphosis with different undergoing processes such as spinning the cocoon, pupation, moulting or pigmentation takes place under this cap and usually pass unobserved (Snodgrass, 1956; Rembold et al., 1980). In the same situation is the reproductive phase of the varroa mite, which is totally protected by the capping barrier, with negative consequences on the honeybee's natural defending mechanisms, such as grooming or hygiene mechanisms, as well as on the treatments' effectiveness.

Studying the brood capping closely, one can observe the presence of the two layers: (1) the external wax layer, applied by worker honeybees in order to protect the larvae from falling down during the pupation process (Siceanu, 1996), and (2) the internal layer, which is represented by the cocoon tissue formed in the pupation process right after capping (Snodgrass, 1956; Rembold et al., 1980). The external surface of the capping made by wax, which has the color of the neighbouring comb cells as an economic strategy of the honeybee colony, is rough and has small openings visible through a stereomicroscope.

However, the internal surface is smooth and glossy-white, with a relative transparency, allowing the wax colour to be slightly visible (Figures 1 and 2).

Figure 1. The external view of the brood cap in worker brood**.** In the green background one can notice small openings in the irregular composition

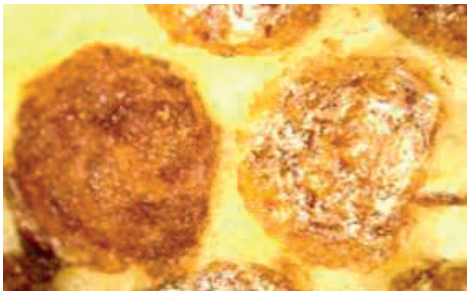


Figure 2. The internal view of the brood caps in worker brood**.** One can notice the white-shiny cocoon layer (right) and the wax layer after the cocoon was removed (left)

This porous, spongy-like structure of the honeybee brood cap, and the property of some organic substances (especially formic acid) to rapidly volatilise and pass through it, have recently led us to develop new procedures (Siceanu et al., 2019), for varroa mite control in capped brood. By their chemical properties (for example the pungent and irritating smell) (Formic acid-technical evaluation report, 2011), the highly volatile organic acids, like formic and acetic acids, affect the varroa mites through various mechanisms such as breathing inhibition (asphyxiation), disruption of the basic metabolic pathways (Rosenkranz et al.,

2010) and very likely by affecting the soft membranes (e.g., apoteles, intersegmental membranes) as well as by impairment of the sensory organs (e.g., pit organ), considering its chemosensing abilities (Nganso et al., 2020; Plettner et al., 2017).

Today, it is also well known that formic acid is the only substance that acts on brood when applied in the whole colony treatment, its effectiveness being very variable as many studies indicate: 41-95% (Calderón et al., 2010), 94.74% (Amrine & Noel, 2007), >60% (vanEngelsdorp et al., 2008). Some research even focused on brood treatment, separately by honeybee colony, for 1-2 hours, with very good results (up to 100% mite mortality) (Calis, 2001; Fries, 1991) and some practical information and applications were tried and recommended (Guido, 2018). The efficacity of formic acid on phoretic mites is also very variable (at least 40% and even over 95%), showing the importance of many factors involved, products or methods used (Pietrapaoli & Formato, 2019; Underwood & Currie, 2005, 2003; Elzen et al., 2004; Feldlaufer et al., 1997; Mutinelli et al., 1994). Most of these authors recommend the treatments of honeybee colonies with formic acid in long application (7-30 days) at the same time with monitoring the external temperature conditions in certain intervals which helps in vaporization control and reduction of the side-effects on bees. Unfortunately, the long duration of formic acid application can harm honeybees, queens, communications between individuals and the general development of the honeybee colony. These phenomena are highlighted in almost all the above-mentioned researches, as well as in practice. To overcome these problems, some new application methods were developed (Amrine & Noel, 2007; van Engelsdorp et al., 2008) to decrease the concentration and treatment duration, as the external temperature can be better predicted. The use of acetic acid in varroa mite control was also considered by researchers, but its effectiveness by whole colony treatment was lower than that of the formic acid (van Engelsdorp et al., 2008). To have a good effectiveness for varroa mite control, the use of highly volatile acids should be a very reasonable solution as they are also cost effective and organic substances. Their use is allowed in varroa mite control in organic beekeeping in the European Union, as it is ruled in Council Regulation 834/2007, Regulation (EU) 2018/848 of the European Parliament.

Taking into account the negative effects of these substances on honeybees it is important to develop new methods of treatment, focusing only on capped brood (drone and worker), where the most part of varroa mite population exists in the active season. At the same time, this approach could be included in the sustainable strategies for varroa mite control which may be applied at any moment during the active season or at key moments, especially before rearing winter honeybees, in order to limit the natural development of the mite population, whose peak overlaps with this period.

Another advantage of limiting the treatment with volatile acids to capped brood combs is represented by a lower risk of honey contamination, having in view their hydrophilic properties and the presence of a higher content in honey, over the normal limits, following the conventional treatments.

In order to help the transfer of the volatile acids into brood cells by decreasing the treatment duration (from days or even hours to minutes), new procedures were developed and tested in our laboratory in recent years (artificial brood decapping, closed boxes using pression, brushing brood) (Siceanu et al., 2019). Following these preliminary researches, we focused on those treatment procedures that could be optimised and practically applied in beekeeping with very good results. Thus, the aim of the present study was to evaluate the effectiveness of two procedures for the capped brood treatment in very short time applications, on the mite (*Varroa destructor)* mortality inside the cells (the reproductive phase).

These procedures use highly volatile acids (formic and/or acetic acids) by (1) natural vaporization and saturation in closed space or by (2) capping brushing. If the first procedure - natural vaporization and saturation in closed space - represents an improved procedure of the time-concentration parameters, following the researches published by Fries in 1991, and by Calis et al., in 2001, the second one - capped brood brushing - represents a completely new

procedure, firstly communicated and registered for patent by Siceanu et al., in 2019.

MATERIALS AND METHODS

1. Experimental design

To test the effectiveness of these treatment procedures, an experimental design was established and varroa mite mortality inside the capped brood, found in all the developmental stages, was assessed.

The applied procedures are based on:

(1) the air saturation with highly volatile acids by natural vaporization in a special airtight box, assuming that a high concentration will naturally and rapidly enter the capped cells, and (2) brushing the capped brood combs directly with the highly volatile acids, using the natural properties of capping to absorb the substance and transfer it into cell for a short time interval.

The experiments were carried out in the 2018-2020 active seasons, in an experimental apiary (Băneasa 2) in the frame of Honeybee Genetic and Breeding Laboratory of the Institute for Beekeeping Research and Development - Bucharest (44°29′33″N 26°04′45″E). We included in the experimental apiary a total of 50 honeybee colonies of *Apis mellifera carpatica* subspecies, with young queens (2018, 2019), managed in Dadant hives on 10 frames. The experimental colonies have not been treated since 2018 in order to increase the level of varroa mite infestation for the 2019-2020 experiments. To increase the probability of having as much as possible a high infestation with varroa mite, for a better effectiveness in varroa mite counting, the procedure applications and the measurements were done from July 15th to August 30th, both in 2019 and 2020. At the same time and for the same reason, the donor colonies for capped brood combs were randomly selected from those with the highest level of infestation, being screened by natural mites that had fallen on the bottom boards. The experimental procedures were applied on honeybee capped brood combs, without adult bees (workers, drones, queen). To evaluate the impact of treatments on different categories of mites, the combs were generally selected to have brood of older ages (6-12 days post capping) in order to find as much as possible all the developmental stages of varroa mite.

A number of 10 combs was treated for each experimental variant according to the experimental design in Table 1 and the mite mortality evaluations were done under laboratory conditions.

As natural infestation of capped brood means, generally, varroa mites in a reproducing status and as they can be easily identified by the presence of white faecal deposits on the cell walls, a certain indicator of live mites (Dietemann et al., 2013; Büchler et al., 2017), control variants were not included to assess its natural mortality in the untreated capped brood. In some similar experiments (vanEngelsdorp et al., 2008; Fries, 1991), the natural mortality of the varroa mite included in tests as control was extremely low. Also, the experiments were designed to include different experimental variants grouped in the two procedures to test the specific variables (substance, time,

Table 1. The experimental design for capped brood treatments by normal vaporization and by brushing the volatile acids

Experimental design and treatment variants	No. of treated combs	Concentration of active substance %	Quantity (ml)
The experimental group to test the first procedure – The capped brood treatment, for different time intervals, in closed space, saturated with formic or acetic acid vapours by natural vaporization			
Formic acid treatment for 15 minutes (T1-FA 5')	10	85	100
Formic acid treatment for 10 minutes (T2-FA 10')	10	85	100
Formic acid treatment for 5 minutes (T3-FA 15')	10	85	100
Acetic acid treatment for 20 minutes (T4-AA 20')	10	99	100
The second experimental group to test the second procedure - The capped brood treatment by brushing with formic and acetic acids of different concentrations			
Brushing with formic acid 85% (T5-FAB 65%)	10	65	-
Brushing with formic acid 65% (T6-FAB 85%)	10	85	-
Brushing with acetic acid 99% (T7-AAB 80%)	10	80	-
Brushing with acetic acid 80% (T8-AAB 99%)	10	99	-
Brushing with a formula based on formic acid 65% and acetic acids 80% in different proportions* (T9-FAAB 65&80%)	10	65&80	-
*formic acid 65%, acetic acid 80%, plant extracts (*Ocimum basilicum, Thymi vulgaris, Mentha piperita, Mellisa officinalis*) and sugar in proportion of 6:2.5:1:0.5.			

concentration), so as to be able to perform comparisons, statistical analysis and data interpretation. The plants used in the extract are medicinal and aromatic plants, containing active substances recognized for positive effect on the honeybee digestive system and anti-repellent effect. The sugar role was to assure a good adherence of formula on the comb surface, to better maintain the formula substances in the porous structure of the cap. Thus, the formula based on formic and acetic acid (FAAB 65 & 80%), as well as some plant extracts and sugar, was specially created to decrease the concentrations of acids, to include the necessary active substances for the best efficacy on varroa mites' mortality, to have a good adherence, as well as to help attract honeybees after treatment to take care of the treated brood in a shorter period of time after treatment.

2. The procedures application.

2.1. The capped brood treatment, for different time intervals, in closed space, saturated with formic or acetic acid vapours by natural vaporization.

Before treatment (at least 10 minutes), an airtight box was prepared, by application of 100 ml formic acid of 85% concentration or acetic acid of 99% concentration on textile elements placed on lateral walls and on the inner cover, so as to sustain a rapid vaporization and air saturation inside the box. As a result of some measurements, the quantity of vaporised formic acid during the treatment of 4 combs, which is the frames capacity of the treatment box in our experiments (including all operations), was between 15 and 30 g at a volume of 33 dm^3.

In order to apply this procedure, irrespective of surface or presence of open brood or food, the worker honeybee capped brood combs to be treated were shaken and brushed off to eliminate the covering bees in the origin colony.

The combs were put into the airtight box, after saturation with formic acid by natural vaporization, they were treated for 5, 10 and 15 minutes. The treated combs were put back into the origin colonies until the next day when the mite mortality was assessed (Figure 3).

2.2. The application of treatment by brushing the capped brood surfaces with tested substances.

To apply this procedure, irrespective of the capped brood surface or the presence of open brood or food, the worker capped brood combs were shaken and brushed off to eliminate the covering bees in the origin colony. The brood combs were successively treated (brushed) with substances of different concentration or formula (Figure 4), depending on experimental variants and put into a ventilated box placed near the original hive (Figure 5).

Figure 3. The application of treatment in closed space, saturated with organic, volatile acids vapours by natural evaporation

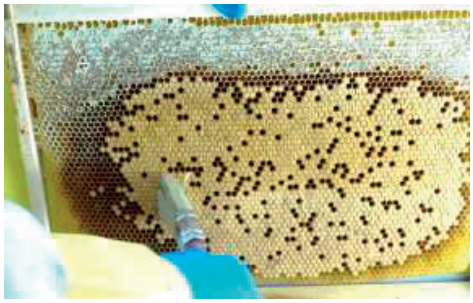


Figure 4. The application of treatment with volatile organic acids by brushing the capped brood

Figure 5. The application of treatment with volatile organic acids by brushing the capped brood by a simplified variant, near the treated hive

The honeycombs were held so that the treatment solution should not leak into the uncapped cells in which there could be eggs,

brood larvae, honey or pollen bread, so as to avoid their contact with acids. To treat the combs with experimental substances, we used a paintbrush with medium stiffness bristles, about 4-10 cm wide. The treatment product was applied and brushed with a light press, to help the cap absorb the tested product. The surface of the capped brood was brushed so that all cells with capped brood were also covered with the treatment substance. The brushing was done with left-right movements, to avoid the accumulation of drops on the lower edge of the uncapped cells and leakage inside them.

To carry out the treatment, the volatile acids were put into a special plastic box which is strongly fixed by the hive wall (Figure 5). The operation was repeated on all capped brood combs' surfaces from the experimental variants. The treated brood was immediately placed in a well-ventilated box hive type (e.g. frames transport box, swarm box, etc.) as shown in the Figure 5. The box was covered with a board, so that the bees could not enter the space (to prevent robbing if there was a risk) and left for 10-15 minutes, during which time, most of the treatment substances evaporated inside and outside the cells.

The treated combs were not immediately returned to the colony because the amount of evaporated acids can harm the honeybees or queens in the honeybee colonies, especially in the first minutes. The direct contact of the testing acids with any individual (bees or queen) can kill them. For this reason, it is recommended to keep the treated combs after brushing in separately boxes for at least 10 minutes, depending on the treated surface, until the excess of substances is evaporated.

The treated combs were put back into the origin colonies until the next day when the mite mortality was assessed.

While using the treatment substances, it is mandatory to wear acid-resistant protective gloves, glasses and mask to prevent inhalation of acid vapours or direct contact. To better understand this procedure, two scientific-technical video-films were developed and openly published (Siceanu et al., 2019; Siceanu, 2020).

3. The measurements on varroa mite mortality inside the capped cells.

To give the treatment time for action, and to assess the impact of treatment on different categories of varroa mite which normally is found in the infested cells, the mortality was assessed on the day following the treatment (24 h). For each application procedure specific data about the treatment was registered (concentration, quantity, time), number of checked cells, number of infested cells as well as number of live and dead mites for each category. Thus, treated combs were taken out of the colony and the number of dead and alive varroa mites (including all individuals in a dying state) was assessed, using a stereomicroscope (Olympus SZ61) with 6,7X-45X magnification.

To do these evaluations, the cells were opened with a tweezer, cell by cell, in rows, following the standard protocol (Dietemann et al., 2013; Büchler et al., 2017) or in some cases using the artificial decapping method to uncap rapidly a larger portion of cells (Siceanu et al., 2018; 1996). As mentioned above, the infested cells were more easily identified by the presence and white aspect of mite dejection on the cell walls. Each pupa from the infested cells were taken out and carefully put on a slide to be inspected. All the categories of varroa mites that were found and their state (dead or alive) were registered. In the same manner, the emptied cells were inspected. The varroa mites counting was assigned to the following different categories of mites according to their aspect: foundress females (FF), adult males (AM), protonymphs - males and females (P), deutonymphs - females (D), and adult daughters (AD) as shown in Figure 6.

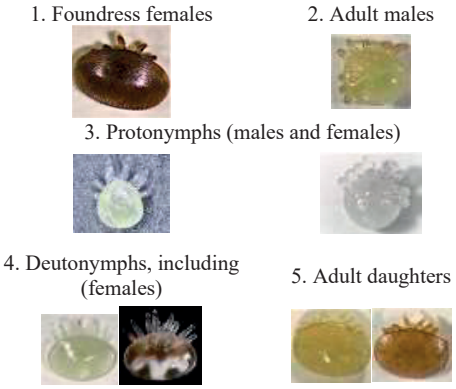


Figure 6. The aspect of different stages of varroa mite development in capped brood (6,7X-45X, stereomicroscope (Olympus SZ 61). Photos© Institute for Beekeeping Research and Development, Bucharest, Honey bee Genetics and Breeding Laboratory

The adult mites and immature stages (eggs, larvae, protonymphs, deutonymphs) present a sexual dimorphism and a gradual sclerotization of the exoskeleton which help their identification.

As it is very difficult, confusing and time consuming to distinguish between protonymphs/deutonymphs of males when compared with protonymphs of females, these stages were included into protonymphs category of males and females, and from the treatment perspective they can be similarly affected as they are individuals of similar size with an unsclerotized exoskeleton.

Deutonymphs received a special attention as their immobile phase (which last 48 h) (Dietemann et al., 2013), can be assigned to death category, the live individuals presenting an internal specific motility which can be noticed by their transparency. To notice these details, the deutonymphs were placed in a good position and light at a 45X magnification.

To perform statistical analyses on the obtained data, the tests for outlier's data identification (Grubbs test) and normal data distributions (Anderson Darling test) were firstly applied. To apply different statistical tests in order to assess the statistical significance threshold of different treatments' effectiveness, we used a Bartlett test for the variances' homogeneity, calculated in R software followed by specific tests to check the averages' homogeneity assumptions (Free software for statistical analysis). Thus, the homogeneity of the averages within each experimental group was analysed by a Welch's ANOVA test for unequal variance followed by a Games Howell post-hoc test in the frame of the first experimental group, and an ANOVA test followed by a Tukey post-hoc test for equal variance in the second experimental group. Data were calculated in Excel Office 2016 worksheets completed by XRealStats and Sigma XL modules, according to the statistical analysis guidelines presented in the literature (Sandu, 1995; Pirk et al., 2013). Additionally, a set of boxplots histograms on different treatments and categories of mites in the frame of the two groups of treatments were presented. It is important to mention that the percentage of varroa mite mortality 24 hours later, following the treatment application, was the response variable in all the statistical analyses.

RESULTS AND DISCUSSIONS

The obtained results regarding the average of varroa mite mortality in the cells (%), assessed at 24 hours after treatments application, in different treatments, are shown in Tables 2, 3, and 4. The results were obtained by evaluating an average of 26.4 single or multiple infested cells per comb, out of 139.3 checked cells per comb in average, per total experiment. The general infestation level of brood combs on average was 19.5% (Table 3). According to these data, a high percentage of varroa mortality (>85%) was registered in more treatments performed by the two types of procedures: FA 10 min, FA 15 min, FAB 65%, FAB 85%, AAB 99%, and FAAB 65 & 80%. Analysing the averages, in the first experimental group (T1-T4), the best effectiveness of brood treatment (Ave. = 97.96%, St err. ± 0.56) was registered after keeping the capped brood combs in the saturated space with formic acid vapours for 15 minutes. A lower effectiveness (Ave. = 85.74%, St err. ± 1.89) was registered at a 10 minutes interval, while a low effectiveness (Ave. = 26.22%, St err. ± 1.44) was registered after 5 minutes of treatment. These data show an increasing effectiveness of the formic acid combating the varroa mite in a saturated space, in a certain time interval (5-15 minutes), with maximum effectiveness at 15 minutes treatment. The effectiveness of acetic acid 99% (Ave = 68.24%, St err. ± 1.27) when used to saturate a treatment space for 20 minutes was lower than that of the formic acid used for 10 minutes.

In the second experimental group (T5-T9) regarding the brushing of capped brood with volatile acids of different concentrations, a high effectiveness (over 90%) of treatments on varroa mite mortality inside the cells was registered in the experimental variants in which formic acid was used: FAB 65% (Ave. = 90.48%, St err. ± 1.29), FAB 85% (Ave. = 92.64%, St err. ± 1.38), and FAAB 65&80% (Ave. = 96.36%, St err. ± 0.84). Acetic acid of 99% and 80%, when used alone in brood brushing, showed a lower effectiveness (AAB 99%: Ave. = 89.68%, St err. ± 0.89, respectively AAB 99%: Ave. = 74.46%, St err. ± 1.88), but a better one than in the treatment in saturated box (AA 20'). For a better overview,

the results of each experimental variant were plotted in Figure 7, highlighting the quartiles repartition and averages of varroa mite mortality as percentage. Thus, one can easily remark the best treatments, also by values repartition on quartiles (75th, 50th and 25th) and overall average of each treatment.

Table 2. The varroa mite mortality percentage in average per each comb, in different experimental variants

Treated brood combs	*The 1st experimental group*				*The 2nd experimental group*				
	T1 FA 5'	**T2 FA 10'**	**T3 FA 15'**	**T4 AA 20'**	**T5 FAB 65%**	**T6 FAB 85%**	**T7 AAB 80%**	**T8 AAB 99%**	**T9 FAAB 65&80%**
C1	22.54	80.90	94.00	64.79	89.63	90.85	81.03	90.91	100.00
C2	32.65	78.43	98.08	64.29	88.27	95.83	82.76	91.85	93.33
C3	29.23	90.14	96.97	72.22	90.00	85.99	70.23	88.71	100.00
C4	25.53	86.61	99.07	71.43	96.10	92.12	78.70	90.72	98.55
C5	17.46	80.43	100.00	76.12	96.23	97.45	75.84	95.05	97.50
C6	26.09	86.67	96.77	68.14	88.24	95.92	66.67	90.00	92.55
C7	27.85	87.37	98.89	65.31	83.33	86.73	67.42	87.39	96.15
C8	22.22	92.50	97.83	67.09	87.95	89.11	78.38	89.47	96.05
C9	27.37	95.65	97.96	63.95	90.43	94.25	70.69	84.42	95.45
C10	31.25	78.69	100.00	69.09	94.64	98.17	72.86	88.30	94.00
Ave.	**26.22**	**85.74**	**97.96**	**68.24**	**90.48**	**92.64**	**74.46**	**89.68**	**96.36**
St. Err. ±	**1.44**	**1.89**	**0.56**	**1.27**	**1.29**	**1.38**	**1.80**	**0.89**	**0.84**

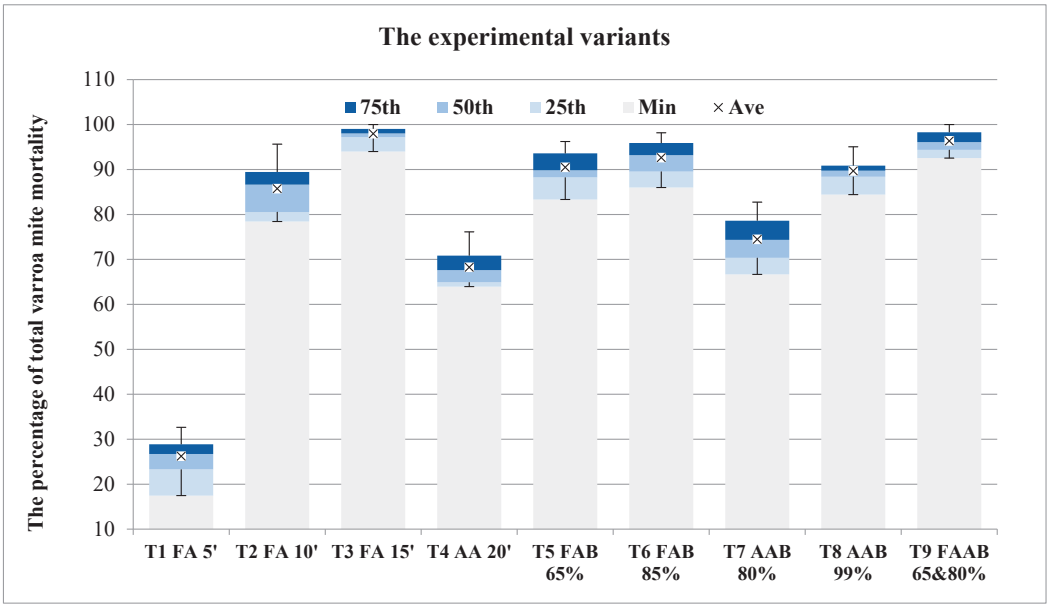


Figure 7. A box plot presentation of varroa mite mortality data (%) in capped brood treated with formic and acetic acids by experimentally tested procedures

Table 3. The obtained results regarding the number of checked cells, infested cells, varroa mites and the average of mortality on each treatment

Treatments	Number of combs	No. of checked cells	No. of evaluated infested cells	Infestation level %	The varroa mite's evaluation		
					Total (dead & alive) (T)	Dead (D)	Mortality % (M)
T1 - FA 5 min.	10	1356	178	13.13	606	158	26.07
T2 - FA 10 min.	10	1866	230	12.33	734	633	86.24
T3 - FA 15 min.	10	1228	168	13.68	763	749	98.17
T4 - AA 20 min.	10	1164	232	19.93	826	560	67.80
T5 - FAB 65%	10	1548	415	26.81	1609	1457	90.55
T6 - FAB 85%	10	1308	420	32.11	1632	1499	91.85
T7 - AAB 80%	10	1394	251	18.01	1189	890	74.85
T8 - AAB 99%	10	861	221	25.67	931	837	89.90
T9 - FAAB 65&80	10	1824	259	14.20	1030	988	95.92
Total	90	12549	2374	18.92	9320	7771	-
Ave.	10	1394.3	263.8	19.5	1035.6	863.4	83.38
St. Err. ± T1-T4	-	*89.85*	*9.52*	*0.98*	-	-	*15.79*
St. Err. ± T5-T9	-	*74.44*	*26.62*	*1.47*	-	-	*3.60*

Table 4. The obtained results regarding the number of varroa mites found in brood (total and dead) as well as its mortality in average (%) on different categories of mites and each treatment

Treatments	*The number of varroa mites found in brood and its mortality on different categories after treatments (at 24 h)*														
	Foundress			Males			Protonymphs			Deutonymphs			Daughters		
	T	D	M %	T	D	M %	T	D	M %	T	D	M %	T	D	M %
T1 FA 5 min.	188	37	**19.6**	82	26	**31.7**	147	42	**28.5**	115	32	**27.8**	74	21	**28.3**
T2 FA 10 min.	235	198	**84.2**	90	80	**88.8**	164	145	**88.4**	151	130	**86.0**	94	80	**85.1**
T3 FA 15 min.	168	164	**97.6**	84	82	**97.6**	215	215	**100.0**	198	193	**97.4**	98	95	**96.9**
T4 AA 20 min.	258	101	**39.1**	93	81	**87.1**	137	124	**90.5**	172	152	**88.3**	166	102	**61.4**
T5 FAB 65%	474	428	**90.3**	223	206	**92.3**	238	230	**96.6**	441	390	**88.4**	233	203	**87.1**
T6 FAB 85%	486	454	**93.4**	215	205	**95.3**	211	209	**99.0**	461	392	**85.0**	259	239	**92.2**
T7 AAB 80%	278	182	**65.4**	169	108	**63.9**	222	196	**88.2**	289	229	**79.2**	231	175	**75.7**
T8 AAB 99%	310	277	**89.3**	145	131	**90.3**	184	175	**95.1**	175	149	**85.1**	117	105	**89.74**
T9 FAAB 65&80	274	261	**95.2**	88	80	**90.9**	297	297	**100.0**	257	240	**93.3**	114	110	**96.4**
Total	**2671**	**2102**	**-**	**1189**	**999**	**-**	**1815**	**1633**	**-**	**2259**	**1907**	**-**	**1386**	**1130**	**-**
Ave.	**296.8**	**233.6**	**78.7**	**132.1**	**111.0**	**84.0**	**201.7**	**181.4**	**89.9**	**251.0**	**211.9**	**84.4**	**154.0**	**125.6**	**81.5**
St. Err. ± T1-T4	-	-	*18.4*	-	-	*15.0*	-	-	*16.3*	-	-	*15.8*	-	-	*15.1*
St. Err. ± T5-T9	-	-	*5.4*	-	-	*5.7*	-	-	*2.0*	-	-	*2.3*	-	-	*3.4*

In order to perform statistical analyses, the data were checked out for outliers' values, using the Grubbs test in Excel Office 2016 worksheets, checked out also by XRealStats software, the obtained results showing the lack of these data.
Further on, the data normality was checked out using the Anderson-Darling test performed in Excel Office 2016 completed by Sigma XL module. The all obtained p-values were greater than the level of confidence (α=0.05) which validate the assumption that the data sampled are from a normal distribution.
To establish the homogeneity of variances of the tested samples, in a normal distribution of data, a Bartlett test performed in R software was performed for equal samples, all treatments and by groups of treatments. The results are presented in the Table 5.

Table 5. The results on variances homogeneity – Bartlett test, equal samples

Treatments	**Bartlett's K-squared**	df	p-value	X^2 critic α=0.05	The results
T1-T9 (all treatments)	17.618	8	0.02428	15.51	unequal variance
T1-T4 (the 1st group of treatments)	10.543	3	0.01447	7.81	unequal variance
T5-T9 (the 2nd group of treatments)	6.8424	4	0.1445	9.49	equal variance

The obtained values and their probability show a heterogenic variance in the tested treatments which is generated by the first group of treatments, as by subsequently testing an unequal variance in the first group of treatments (K-squared > X2 critic, at α=0.05) and an equal variance in the second group of treatments was found.
To continue with the statistical analysis on the first group of treatments, a Welch's ANOVA test assuming unequal variance was applied to establish if the differences would be identified also concerning the treatments' averages.

Table 6. The Welch's ANOVA test of averages assuming unequal variances for the 1st group of treatments T1-T4

Welch's ANOVA test	Numerator df	Denominator df	F-calc.	Probability level
Between Groups	3	17.87	735.4	6.59E-19
F-critic (df 3; 18; α= 0.05) =3.16 F-critic (df 3; 18; α= 0.001) = 8.49 The result. F calc> F crit. The null hypothesis of equal averages is rejected				

The summarised results in the Table 6 show highly significant differences between the averages of the 1st group of treatment.
As a result, a Games-Howell post-hoc test was applied further on to establish the statistical significance of differences between the averages of treatments, grouped two by two. The results are presented in Table 7.

Table 7. The pair-wise comparison assuming unequal variances and equal samples (Games-Howell post-hoc test) for the first group of treatments T1-T4 (XRealStats)

Games-Howell test		Ave. diff.	q-calc.	df	q-crit α=0.05	p-val.
T1 FA 5'	T2 FA 10'	59.5	35.4	17	4.02	1.14E-13
T1 FA 5'	T3 FA 15'	71.7	65.6	12	4.20	-4.4E-13
T1 FA 5'	T4 AA 20'	42.0	30.9	18	4.00	1.66E-13
T2 FA 10'	T3 FA 15'	12.2	8.7	11	4.26	0.00039
T2 FA 10'	T4 AA 20'	17.5	10.8	16	4.05	5.72E-06
T3 FA 15'	T4 AA 20'	29.7	30.2	12	4.2	1.96E-10

As it can be easily noticed, there are highly significant differences between all treatments when compared two by two, highlighted by the pairwise average difference where q-calculated is higher than q-critic at a confidence level α=0.05. The lowest difference can be remarked between the 10 and 15 minutes treatments when formic acid was used.

Table 8. The results on averages' homogeneity (ANOVA single factor test), used for test the equal samples and equal variances for the 2nd group of treatments T5-T9

ANOVA single factor test					
Source of Variation	*SS*	*df*	*MS*	*F calc.*	*P-value*
Between treatments	2811.7	4	702.95	42.19	1.11E-14
Within treatments	749.73	45	16.66	-	-
Total	3561.5	49	-	-	-
F-critic (df 4; 45; α= 0.05) =2.61 F-critic (df 4; 45; α=0.001) = 5.70 F calc > F crit. The null hypothesis of equal averages is rejected					

The results of ANOVA single factor test, presented in Table 8, show highly significant differences between all treatments as F calculated is higher than F critic (α=0.001).
To statistically compare the treatments in the second group of treatments we used a one-way ANOVA test followed by a Tukey post-hoc test. Comparing the different brushing treatments by Tukey post-hoc test, to determine

if at least one group of averages is different from the others, the following results (Table 9) were obtained:

Table 9. The pair-wise comparison assuming equal variances and equal samples (Tukey post-hoc test), for the second group of treatments T5-T9

Tukey test	T5-T9	T5-FAB 65%	T6-FAB 85%	T7-AAB 80%	T8-AAB 99%	T9-FAAB 65&80%
T5-T9	Ave.	**90.48**	**92.64**	**74.46**	**89.68**	**96.36**
T5-FAB 65%	**90.48**	0	*2.16 (NS*)*	*-16.02 (HS)*	*-0.80 (NS)*	*5.88 (S)*
T6-FAB 85%	**92.64**	0.761	0	*-18.18 (HS)*	*-2.96 (NS)*	*3.72 (NS)*
T7-AAB 80%	**74.46**	2.58E-10	5.84E-12	0	*15.22 (HS)*	*21.90 (HS)*
T8-AAB 99%	**89.68**	0.992	0.491	1.09E-09	0	*6.68 (HS)*
T9-FAAB 65&80%	**96.36**	0.019	0.265	2.46E-14	0.005	0
w-critic (tab) = q (df 5; 45; α=0.05) = 5.21 w-critic (tab) = q (df 5; 45; α=0.01) = 6.36						
**NS – Non-significant differences; S - Significant differences; HS - Highly significant differences.*						

This statistical test shows us that the varroa mite mortality registered non-significant differrences (NS, w calculated < w critic, at α=0.01) between the following brushing treatments:

- formic acid 85% and formic acid 65% concentration;
- formic acid 85% and formula based on formic and acetic acid (65&80%);
- formic acid 85% and acetic acid 99%;
- formic acid 65% and acetic acid 99%.

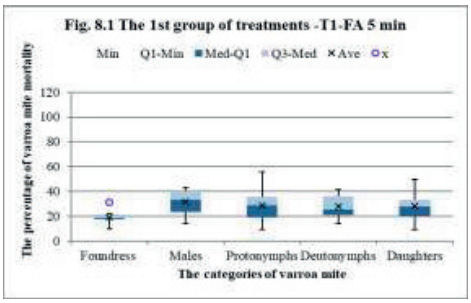
Comparing the treatments based on formic acid 65% with the formulation based on formic and acetic acids we registered significant differrences (S) in varroa mortality at the level of confidence α=0.05, but no differences at α=0.01.

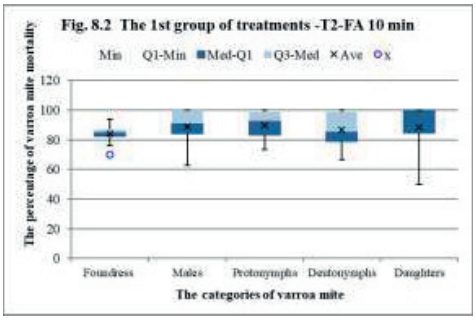
Highly significant differences in varroa mite mortality were found when the treatment formula was compared with acetic acids-based treatments, but important differences were found also between the two acetic acid-based treatments.

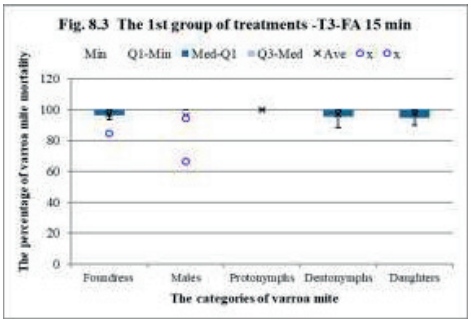
Highly significant differences were found also when acetic acid 99% was compared with formula based on formic and acetic acid, but at a lower level (w = 6.68, w calc at α at 0.01 = 6.36).

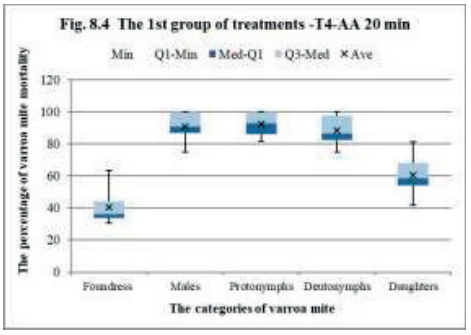
Regarding the different categories of varroa mite mortality in the brood cells (at 24 h) following the two procedures of treatment, the results on their mortality and standard error (±) for each treatment are presented in table S2.

For a better image of the data obtained on each treatment (n=10 combs), box plots with quartiles, medians and averages as well as their limits of variation are presented in Figures 8.1 - 8.9.

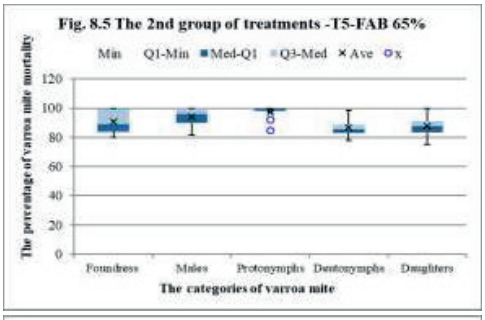


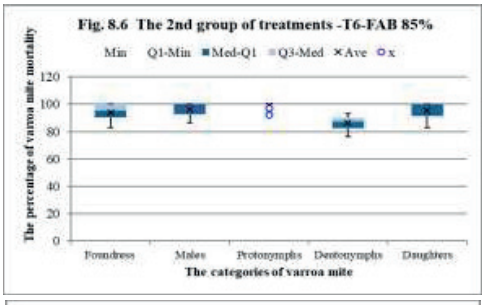


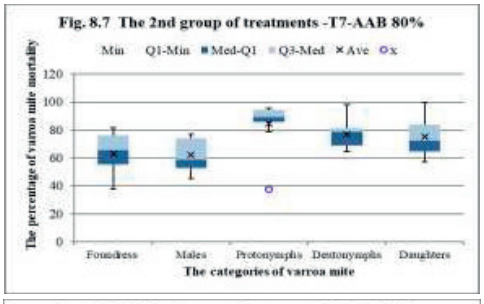


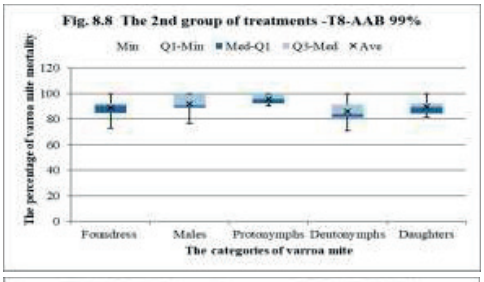


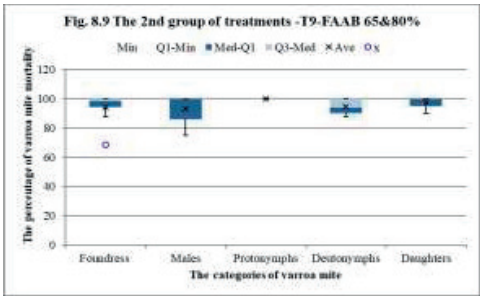
Figures 8.1-8.4. Boxplots on different categories of varroa mite mortality in honeybee brood treated with formic and acetic acids by natural vaporization in closed space procedure











Figures 8.5-8.9 Boxplots on different categories of varroa mite mortality in honeybee brood treated with formic and acetic acids by brushing procedure

These figures show that in the 1st group of treatments, the formic acid act almost equal on different varroa categories, but the treatment duration is very important on the mortality level, while acetic acid act better on immature and unsclerotised varroa individuals. In the 2nd group of treatments one can notice that there is a better and more similar effectiveness on all varroa categories in using both active substances (formic and acetic acid) with lower values when using acetic acid alone and in lower concentration.

The results we have obtained validate the hypothesis that the new tested treatment procedures are very effective (up to 100%) in treating *Varroa destructor* mite in the capped brood of the honeybee colonies, in short applications (minutes), severely interrupting the reproductive phase of varroa. However, the heterogenic variances and averages in the 1st group of treatments shows that the time parameter as well as the different volatilization properties of the two substances are very important in performing capped brood treatments in acid-saturated spaces, influencing the percentage of varroa mite mortality in the capped brood. Thus, the obtained results show us the importance of a minimal treatment duration, for acid molecules to penetrate the caps and make contact with the different categories of mites to have an immediate high mortality. This experiment shows us that, when the formic acid is used, it is important to keep the combs in the saturated boxes for a minimum of 10 minutes to have at least an 85% immediate mortality of varroa mite inside cells. In the second group of treatments, all the experimental brushing treatments having formic acid in their composition registered very good results on mortality of varroa mites. The best effectiveness was obtained with the formic acid of an 85% concentration or when the formula based on formic and acetic acid was used, but insignificant differences were registered between all treatments based on formic acid (65%, 85% and formula). Good results (on average an 89% mortality) were registered also when the acetic acid of 99% concentration was used and insignificant differences were found when it was compared with the formic acid 65% and 85%. The obtained results are better in the case of

brushing procedures as once the capping is imbued, a part of the substance will immediately penetrate the cap and will fill the space of cells. As in the first group of treatments, the formic acid used by brushing procedure was proved to be more effective than acetic acid in order to obtain an immediate mortality, evaluated at 24 h after treatments.

According to the mortality level of different categories of varroa mite, the obtained results in the first group of treatments, where acid concentration varies with the treatment time (minutes) and the substance used (formic acid as compared with acetic acid), one can notice that adult females are the most resistant category to the treatment, especially when acetic acid is used, while the immature mites (protonymphs and deutonymphs) are more sensitive, especially protonymphs. This sensitivity depends most probably on the level of vapours (acid concentration) entering the cells and sclerotization degree of their body. The lack of sclerotization in immature stages of varroa brood is an important advantage in these treatments, especially if we want to decrease the time-concentration-dose parameters in the different treatment formula of current procedures.

Deutonymphs stages registered lower values because of the immobile phase which shows a greater resistance to volatiles, as in the case of the pupal stage in honeybees. This resistance can be noticed by observations done on the following stage - the freshly transformed daughters, which could be found live at the evaluation moment, on the next day after treatment. Being very effective in rapidly killing the mites, even the most resistant individuals (adult females), the use of formic and acetic acids in honeybee brood treatments can be considered safe for risks of resistance that these mites could develop, the organic volatile acids being recognized to pose minimal risks (Rosenkranz et al., 2010).

It is important also to mention different observations done during the evaluations:

- the most part of live varroa mites at the evaluation moment looked to be affected by these treatments, as a lower vitality was noticed during the evaluations.
- in some re-evaluations done two or three days after treatment, in the case of effective and very effective treatments (over 70% mortality), the adult females of varroa which remained alive were not capable to continue reproduction; they were found in a dying state, and the eggs were not present inside the cells anymore.

Consequently, from the varroa mite mortality evaluation perspective, we consider that the best moment for the evaluation of the treatments' effectiveness should be done at 2-3 days after the treatment, if there is no purpose to identify the different categories of mite progeny. After this period, the dead protonymphs and deutonymphs are in a decomposing stage and sometimes cannot be identified anymore, while the apparently live varroa mites on the first day after treatment as well as its reproduction activity can be clearly evaluated.

The life cycle of varroa mite would be seriously affected if the foundress is dead or in a dying state and its reproduction and offspring care (e.g., preparing the feeding site) will be affected, too. The same situation would be if the male is dead because the daughters, in case of survival (resulted from immobile deutonymphs) will not have been mated. Even if the viability of honeybee brood was not the purpose of this research, specific experiments being necessary, it was obvious to notice during the experiments that the pupal period was not affected by treatments, continuing its normal development. In these experiments, all the honeybees that emerged from the treated brood were found active and healthy, the hive population and activity being normal during the whole period of experiments. As we noticed, only the mobile stages found in the cocooning, pupation and emerging moments were found to be affected and only the individuals that passed through these stages in the interval of time that the brood was exposed to the substances, and these observations have already been documented even on a longer exposure – 1-2 hours (Calis, 2001; Fries, 1991). According to our observations as well as from older research (Siceanu, 1996), the honeybee pupal stages are more resistant to different factors than larval stages, especially when compared to open brood that requires regular feeding. In the capped brood period, only the nest temperature and humidity are important to the whole transformation from prepupa to adult honeybee. The scientific literature (Ruttner, 1980) shows

that the honeybee brood, both larval and capped brood, if put outside the hive (not in sunlight) for a couple of hours or even more, is relative highly resistant. Thus, in the brood protection perspective, the brushing procedure can be considered superior to the treatment in a closed space as the volatile substances will come in contact only with the capped stages of honeybee brood and the mites inside cells, while in treatment boxes all brood, including larvae and eggs are treated and open brood is clearly affected. Having an immediate result and being targeted only on the capped brood frames, the effect of any external temperature and humidity do not influence the results and procedures' effectiveness as in the classical treatments with formic acids. More than this, by these new treatments we can avoid exposing the adult honeybees which are very sensitive to these substances, as their volatility is very high, increasing rapidly at high, external and nest temperatures.

Currently, at an international level, the treatment of capped brood with organic volatile acids is not practically used, the only method discussed in the literature and proposed in practice being the treatment in closed space (airtight box) for 1-2 hours (Guido, 2018; Calis, 2001; Fries, 1991). Shortening the time of treatment in boxes and developing totally new, minimally invasive and practical procedures such as brushing capped brood with effective volatile substance, would help beekeepers maintain a better control of varroosis. By enlarging the application period and choosing the key moments in the season, especially at the beginning of the season and before "winter bee" rearing, when the surface of capped brood is smaller, to minimize the workload or to combine with different local techniques whenever nest management is necessary (Siceanu, 2020), it is possible to increase substantially the benefit of this application and its effectiveness in combating varroa mite. For example, in the temperate season, the treatment may be done at any moment of the active season, when there is an intervention in the brood nest, even just before or during honey flows, as these substances do not contaminate the honey as well as all the other bee products, especially when applied by these procedures.

Actually, the majority of these treatments are done at the end of the summer season (e.g., August-October for the northern hemisphere, in temperate climate) when the honeybee colony population decreases and the mites' population increases and concentrates itself on the last brood and winter honeybee.

However, to drastically reduce the infestation level and disturb the population dynamic of the mite, the following key moments for applying these treatment procedures would be:

1. Apply early in the spring when there are small areas of capped brood, and the beekeeper performs some inspections or operations for reorganizing the nest (reduction or enlargement). Preferably, the treatment should be done before the beginning of drone rearing if the weather allows the interventions into the hive.
2. Apply when the artificial swarms are established using capped brood, usually with 1-3 frames of capped brood. This is an important treatment in order to give a clean start to the new colony, as usually a lot of varroa mites are taken out together with the capped brood.
3. Apply in the summer, just before the period of "winter bees" rearing, to produce healthy bees under a very low infestation. This can be done easily in the periods when there is a honey flow and the brood surfaces are reduced because the honeybees block the nest with honey, usually the beekeepers are forced to make room for egg laying to obtain bees for wintering.

Taking into consideration the 8-10-fold higher infestation rates of drone brood compared to worker brood, the treatment could be applied on all drone brood surfaces, which highly increases the effectiveness of overall treatment as well as the health of drones and reproduction biology.

In this concept of treatment, in order to kill also the phoretic varroa mites, two options could be available:

1) a classical treatment of honeybee colony with a rapid effect in the same period with brood treatment (e.g., the day before or after a brood treatment);
2) a second brood treatment with formic or acetic acids can be applied after 9-12 days from the first treatment, a necessary interval of time to allow most part of

phoretic mites (foundress females) enter the brood (before capping).

Decreasing the treatment duration and the concentration in active substances as well as the optimization of application procedures during normal inspections, are objectives for further investigations, in order to stimulate beekeepers to apply the capped brood treatment as well as to better protect the honeybee colony, brood and hive products.

Going further with the application possibilities, the new approach could be an effective treatment tool also in combating *Tropilaelaps* sp., taking into account the similarities regarding the reproductive and phoretic phases of these parasites, with a much shorter phoretic phase which contributes to the ineffectiveness of other treatments used in varroa mite control (Pettis et al., 2017; Raffique et al., 2012).

CONCLUSIONS

The two procedures using short time treatments with organic volatile acids are very effective in combating *Varroa destructor* mite in the reproductive phase, interrupting its life cycle.

According to the obtained data, a very high effectiveness of treatments (>90% mortality) was registered in four out of the nine experimental variants, at 24 h evaluation:

(1) the 15 minutes treatment of caped brood in saturated boxes with formic acid;
(2) the treatment of caped brood by brushing with formic acid of 85% concentration;
(3) the treatment of caped brood by brushing with formic acid of 65% concentration;
(4) the treatment of caped brood by brushing with a formula based on formic acid of 65% concentration and acetic acid of 80% concentration.

A good effectiveness (>85% mortality) was also registered in other two experimental variants:

(1) the 10 minutes treatment of caped brood in saturated boxes with formic acid;
(2) the treatment of caped brood by brushing with acetic acid of 99% concentration.

Both formic and acetic acids proved to be effective in saturated space, but their concentration is an important factor when used.

For the first group of treatments, a 10 minutes treatment with formic acid in closed boxes should be sufficient, but further studies could better establish the optimum time-concentration variables. The new procedure of targeted capped brood treatment by brushing could be appreciated as better as compared with saturated space procedure as it does not affect the larval open brood, being a minimally invasive procedure especially with an optimised acid concentration formula. It valorises the natural property of caps to absorb and transfer the volatile organic substances into the cells, transforming its barrier role in a support for substances.

The effectiveness of new, optimal treatment formula for interrupting the life cycle of mite could be better evaluated after several days, when the reproductive success, live status and resistance of individuals can be better evaluated.

By applying the brood treatments in the key moments of the season, even earlier in the active season, and understanding the varroa mite-honeybee colony population dynamic, the level of infestation will decrease substantially, as well as the risks of colony collapsing in the inactive season.

By using the brood treatment and having in view the formic and acetic acids' property of rapid vaporization, the honey bee colony and by-products are not exposed to contamination substances, their impact being limited only to treated combs for a very short time period.

The present approach of brood treatment could open new ways to practical, flexible, organic and cost-efficient treatments in combating varroa mite in the world-wide beekeeping, in obtaining clean hive products for daily consume or apitherapeutic use, as well as in the multifactorial studies which aim to better study and explain the honeybee colony losses.

ACKNOWLEDGEMENTS

This research was funded by Ministry of Agriculture and Rural Development, The National Sectorial Plan ADER 2019-2022, project no. 12.1.1 /13.12.2019 and by Institute for Beekeeping Research and Development Bucharest - Romanian Beekeepers' Association.

We are grateful to Prof. univ. Horia Grosu and Eng. Mircea-Cătălin Rotar for the important support in the statistical analysis of data.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

SIRE GENETIC EFFECTS ON HEALTH TRAITS IN UN-WEANED DAIRY CALVES-PRELIMINARY RESULTS

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Abstract

The aim of the current study was to evaluate the effects that sire-bulls have on health traits of un-weaned dairy calves. The study was carried out at the Experimental Farm of the Research and Development Institute for Bovine, where health data was collected for two consecutive years, between November 2017 and October 2019, following a number of 119 purebred Romanian Black and White calves, managed under identical conditions. Calves were separated into 7 sire-groups, each one of the bulls having at least 10 descendants born and weaned, the maximum descendants from a sire were 35 calves. The incidence of colibacillosis was influenced by the sire of the calves as following: three calves groups did not developed this disease, one group showed a low susceptibility, one group presented an intermediary genetic predisposition, while two groups showed a significant susceptibility ($p \leq 0.10$). Health traits such as rickets, respiratory disease and haemorrhagic enteritis incidences in some sire-descendants groups showed low or lack of susceptibility. Current preliminary results suggest that paternal genetic effects could influence the health of un-weaned dairy calves.

Key words: *cattle, dairy calves, health, paternal genetic effects.*

INTRODUCTION

It is well documented that maternal genetics in cattle can influence the health of animals and their offspring (Berry et al., 2011). Moreover, increased selection of dairy cows for higher milk yields has led to a significant decline in general fitness and health traits, this being observed particularly in high yielding breeds such as the Holstein-Friesians (Oltenacu and Broom, 2010; Parker Gaddis et al., 2014). For instance, breed discrepancies regarding the susceptibility to respiratory diseases have been confirmed by Snowder et al. (2005), studying 12 cattle breeds and reporting a pre-weaning respiratory disease resistance, heritability ranging between 0.00 and 0.26. Furthermore, Berry et al. (2011) reported maternal heritabilities ranging between 0.00 and 0.13 for health traits in dairy cattle, with no previous estimates for paternal genetic effects.

In the recent years, Nordic European Countries developed new breeding programs, which include traits such as survival of both calves and cows (Snowder et al., 2005). Limitations to include disease resistance into breeding schemes for dairy cattle are due to aspects such as lack of genetic variations in some breeds, interrelationships between different diseases, lack of reliable data for some diseases and the economic implications, as previously reported by Stear et al. (2001).

It is of utmost importance to include in the selection of artificial insemination (AI) breeding bulls with the health traits of their progeny, given that high estimated breeding value (EBV) bulls can sire over 200.000 descendants, which could lead to great economic losses if their descendants have high mortality rates or impaired health.

Furthermore, given the new genomic selection developments and the wide use of genotyping protocols, most European countries are using genotyped bulls for AI, considering the potential implications of some recessive genes.

For instance, bovine leukocyte adhesion deficiency (BLAD), which is autosomal recessive disease characterized by recurrent bacterial infections, delayed healing and reduced growth in calves, was found to have an

incidence ranging between 4% and up to 18% in Holstein bulls (Nagahata, 2004). The aim of the current study was to evaluate the effects that genetics of sire-bulls have on health traits of un-weaned dairy calves.

MATERIALS AND METHODS

Animals and general management

The study was carried out at the Experimental Farm of the Research and Development Institute for Bovine Balotesti Romania, where health data was collected for two consecutive years, between November 2017 and October 2019, following a number of 119 purebred Romanian Black and White (Holstein Friesian group) calves (57 males and 62 females, respectively), managed under identical conditions.

Immediately after birth, the calves were separated from their dams and were housed individually in a maternity compartment for the first 10 days of life. Colostrum was administrated in the first 4 hours after birth by an animal caretaker, using a bucket calf feeder. During their first 3 days of life, the calves received minimum 4 litres of colostrum per day, divided into two equal meals at intervals of 12 hours. The following 7 days, they received two meals per day consisting of 3 litres of milk per head. At the age of 10 days, the calves were moved to an outdoor individual hutch with straw bedding, where they were fed with milk replacement, 6 litres/day/head.

The calf diet was supplemented with *ad libitum* starter concentrates (18% crude protein) and alfalfa hay until the age of 3 months, when weaning occurred.

Anthrax vaccination was done at the age of two months and vitamin therapy was applied only to the underdeveloped and ill calves. The management and feeding of calves are similar with the data published in Irimia et al. (2020).

The research activities were performed according with the European Union's Directive for animal experimentation (Directive 2010/63/EU).

Data collection and statistical analysis

Calves were divided into 7 sire-groups, each one of the seven bulls having at least 10 descendants born and weaned within the farm, the maximum descendants from one sire were 35 calves (see Table 1).

Table 1. General experimental design and number of calves per sire-group

Location	44°36′46″N 26°4′43″E	Sire code	Group	Number of descendants
Altitude of site	97 m	ROXX137	I	13
Average rainfall	555 mm	ROXX573	II	13
Summer temperature	22.3°C	ROXX953	III	11
Winter temperature	-3.3°C	ROXX018	IV	10
Weaning age	90 ± 5 days	ROXX019	V	16
Concentrates	18% CP, *ad libitum*	ROXX147	VI	35
Veterinary care	Anthrax vaccination	ROXX341	VII	19

Health trait data was recorded by the farm veterinarian and technicians, with the following health pathologies being reported in calves up to the age of weaning: colibacillosis, coccidiosis, rickets, neonatal calf enteritis, bovine respiratory disease, haemorrhagic diarrhea.

In order to assess the effect of the sire-group on health traits of their calves offspring, the MiniTab®18 software was used (computing average incidence ± standard error of the mean), with the differences between groups being calculated using the Mann-Whitney nonparametric test, with the statistical significance level set at values of $p \leq 0.10$.

RESULTS AND DISCUSSIONS

Incidence of colibacillosis was influenced by the calves sire, with three calves groups out of the total seven not developing this disease (sire-groups I, II and VII), one group showed a low susceptibility to colibacillosis (VI), one group presented an intermediary genetic predisposition (III), while two groups showed a significant susceptibility (IV, V) of $p \leq 0.10$ and

p≤0.05, respectively, when compared to the low susceptibility group (Table 2). Current results are in accordance with those previously reported by Bashahun and Amina (2017), who found a prevalence of up to 100% in some commercial dairy farms, outlining that 20% of the total death losses in un-weaned calves are caused by colibacillosis. Also, Irimia et al. (2020), found that year-factor influences significantly the colibacillosis incidence in un-weaned calves.

Coccidiosis incidence was not influenced (p>0.10) by the sire of calves (Table 2), although slight differences were observed among the calves groups, with incidences ranging from 23.1 ± 12.20 and 53.8 ± 14.40. Lack of statistical Considering that vitamin D was found to play an important role in the metabolism of Ca (calcium) and P (phosphorus), involved in the synthetisation of bones (Sahay and Sahay, 2012) and, also, that it has an important effect on immunity and cell differentiation (Nelson et al., 2012).

One of the most common causes of calf death is represented by neonatal enteritis, given the high prevalence of pathogenic agents such as rotavirus, coronavirus and *E. coli* bacteria in cattle farms (Abd-Elrahman, 2011). Over 50% of all neonatal enteritis cases appear during the first 7 days after birth, with less than 20% occurring after the 2nd week of life (Bendali et al., 1999), although, exceptionally, the highest prevalence of rotavirus is seen between 2nd and 4th weeks after birth (Nourmohammadzadeh et al., 2012). In our study, although slight differences were observed among the calves groups, neonatal enteritis incidence (p>0.10) ranged from 20.0 ± 13.30 to 37.5 ± 12.50 (Table 3).

Table 2. Mean ± SEM for colibacillosis, coccidiosis and rickets incidences among sire descendants

Pathology	ROXX137 [I]	ROXX573 [II]	ROXX953 [III]	ROXX018 [IV]	ROXX019 [V]	ROXX147 [VI]	ROXX341 [VII]
Colibacillosis (%)	0.0±0.00	0.0±0.00	18.2±12.20^{a}	30.0±15.30^{b}	31.3±12.00^{b}	8.57±0.48^{a}	0.0±0.00
Coccidiosis (%)	23.1±12.20^{a}	53.8±14.40^{a}	27.3±14.10^{a}	50.0±16.70^{a}	31.3±12.00^{a}	45.7±0.85^{a}	42.1±1.16^{a}
Rickets (%)	0.0±0.00	0.0±0.00	9.0±9.09^{a}	20.0±13.30^{a}	18.8±10.10^{a}	8.57±0.48^{a}	0.0±0.00

Rows with different superscript differ significantly at p≤0.10
SEM Standard error of the mean

significance could be attributed to the low number of calves within each sire group. Our results regarding the incidence of coccidiosis in un-weaned calves are similar with those previously published by Habtamu et al. (2020) for dairy claves reared in China.

Concerning rickets incidence (p>0.10), nevertheless, three of the calves sire groups did not develop rickets, two calves groups had a slight predisposition to develop rickets of 8.57 ± 0.48% and 9.0 ± 9.09%, while two sire groups had an incidence ranging between 18.8 ± 10.10% and 20.0 ± 13 (Table 2).

Unfortunately, there was no possibility to compare our results for the reason that no previously published data on rickets incidence in calves were found. Moreover, studies on factors that cause rickets in dairy calves are scarce, except general recommendations on dosage for vitamin D in their diets and sun exposure. One of the most common causes of calf death is represented by neonatal enteritis, given the high prevalence of pathogenic agents such as rotavirus, coronavirus and *E. coli* bacteria in cattle farms (Abd-Elrahman, 2011). Over 50% of all neonatal enteritis cases appear during the first 7 days after birth, with less than 20% occurring after the 2nd week of life (Bendali et al., 1999), although, exceptionally, the highest prevalence of rotavirus is seen between 2nd and 4th weeks after birth (Nourmohammadzadeh et al., 2012). In our

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Gulliksen et al. (2009) reported that diarrhea and respiratory diseases increase the risk of death in newborn dairy calves, causing 50% and 15% of the total calves losses, respectively.

Prior reports on the heritability of respiratory diseases susceptibility in both dairy and beef cattle showed low genetic additive effects, however, breed discrepancies have been outlined. For instance, Braunvieh cattle breed appears to be the most susceptible, while crossbred animals have highest resistance (Muggli-Cockett et al., 1992; Snowder et al., 2005). A study conducted in Australia comparing *Bos taurus* (European breeds) with *Bos indicus* (e.g. Santa Gertrudis and Santa Gertrudis crossbreds) cattle for bovine respiratory disease incidence concluded that *Bos taurus* have a greater risk for developing such pathologies compared to indicine cattle (Cusack et al., 2007).

Moreover, a study conducted by Muggli-Cockett et al. (1992) found that calves born from younger dams have a higher pre-weaning incidence of respiratory diseases, which the authors attributed to the lower antibody levels in colostrum of primiparous cows. The significance in maternal transfer of immune factors throughout colostrum was further supported by a trial of Ganaba et al. (1995) that found that throughout vaccination of the dams prior to calving, there is a decrease in respiratory disease incidence in pre-weaned calves.

Un-weaned calves diarrhea was found to be one of the most common diseases in young cattle, causing important economic and productivity losses to the bovine industry at a global level. Differences among prevalence rates between reports were attributed to geographical and animal-management variations (Cho and Yoon, 2014). Furthermore, bovine viral diarrhoea virus was demonstrated to play an important role, in terms of immunosuppression and synergistic effects with other pathogens, for instance having a primary interrelation with pneumo-pathogens. In recent years it was found that bovine coronavirus has a major role in bovine respiratory disease (Jared et al., 2010).

In our study respiratory disease and hemorrhagic enteritis were found exclusively among the descendants of a single sire (group VI), therefore statistical comparison was not possible to conduct (Table 3).

Table 3. Mean ± SEM for neonatal enteritis, respiratory disease and hemorrhagic enteritis incidences among sire descendants

Pathology	ROXX137 [I]	ROXX573 [II]	ROXX953 [III]	ROXX018 [IV]	ROXX019 [V]	ROXX147 [VI]	ROXX341 [VII]
Neonatal Enteritis (%)	30.8±13.30[a]	23.1±12.20[a]	0.0±0.00	20.0±13.30[a]	37.5±12.50[a]	31.4±7.96[a]	36.8±11.4[a]
Respiratory Disease (%)	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	5.7±3.98	0.0±0.00
Haemorrhagic Enteritis (%)	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	5.7±3.98	0.0±0.00

Rows with different superscript differ significantly at p≤0.10
SEM Standard error of the mean

Worth mentioning is the fact that sire group VI had the highest number of calves, accounting for 35 animals (Table 1), and therefore the reliability of our data is higher, compared to sire groups with lower number of descendants.
Diseases in un-weaned calves have multifactorial causes and represents the result of interactions among different factors that could influence to the build-up of infections, including immunological, nutritional, genetic and environmental factors. The study of un-weaned calves diseases is very important because general health represents a key issue of tackling and balancing diverse factors such as pathogens, the underdeveloped immune system of the young animals, environmental aspects and the rearing management practices.

CONCLUSIONS

In the current study, the genetic component of the sire had a significant statistic influence on colibacillosis incidence and susceptibility in un-weaned dairy calves. Moreover, on health traits such as rickets, respiratory disease and hemorrhagic enteritis incidences some groups sire-descendants showed low or a lack of susceptibility.
The current research paper represents a preliminary study. Given the low number of calves for each sire-group available in the current study, which is far less than desired for statistical analyses, larger scale studies are required to include a greater number of bulls with higher number of descendants.
Current study suggest that paternal genetic effects could influence the health of un-weaned dairy calves, nevertheless this research paper gives us only preliminary results.

ACKNOWLEDGEMENTS

This work was supported by a grant of the Romanian Ministry of Agriculture and Rural Development, project number ADER 8.1.5/2019.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

BREEDING AND PRODUCTION PERFORMANCES OF MUSCOVY DUCK LINES

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Abstract

This paper has described breeding and production performances of three Muscovy duck lines (spotted, white and black) during December 2019-June 2020 which has been raise on the teaching waterfowl farm Moara Domnească of the University of Agronomic Sciences and Veterinary Medicine of Bucharest and results are compared with those recorded in year 2001. Each line group has been of 55 birds in size. There were monitored weekly, monthly and for whole laying cycle the following parameters: body weight, egg production, egg weight, fertility, hatchability, egg weight and day-old body weight. Spotted line had an average body weight of 2427 g for females and 4566 g for males an egg production of 59.42 eggs/bird and an egg weight of 78.21 g and a fertility of 88.48% and a hatchability of 39.79% and a day-old body weight of 47.10 g. White line had an average body weight of 2580 g for females and 4450 g for males and an egg production of 72.06 eggs/bird and an egg weight of 76.56 g and a fertility of 86.00% with a significantly lower hatchability (35.80%) and a day-old body weight of 48.32 g. Black line had an average body weight of 2262 g for females and 4750 g for males and an egg production of 52.45 eggs/bird and an egg weight of 84.72 g and a fertility of 90.24% with a significantly higher hatchability (40.90%) and a day-old body weight of 49.92 g.

Key words*: body weight, fertility, hatching, Muscovy duck.*

INTRODUCTION

Waterfowls are on second place next to chickens in poultry meat production with 6.98 million tons produced in 2017. Nowadays there are raised over 1150 million ducks and over 370 million gooses worldwide and flocks are increasing spectacularly in some Asian countries. Worldwide yearly growth was about 2.70% during 2000-2017 which means that total growth was 45.82% with a very good growth rate between 2005-2010 (4.02%/year) (Watt Poultry Statistical Yearbook, 2019).

Worldwide duck meat consumption/capita is of 600g by year and rising with 3.4% by year. Most ducks (83.5% of worldwide production) are being in Asia with 79% of them being raised in China (where consumption/capita is of 2 kg). It has been noticed a tendency to an impressive improvement of following characters: growth rate, feed usage efficiency, reducing fat percentage and increasing breast yield (Linden, 2015; Marin et al., 2015; Popescu-Micloşanu, 2004).

Muscovy duck is very popular especially in France (Guy, 2013) where several researches have been performed (Leclerq and Carville, 1986). Meat is having less fat and a dark red color. Breast weight is about 700 g and this contains about 70% of commercial value of duck and on the other hand thighs and drumsticks are representing 27% (Guy, 2013). Males are raised up to 4.5-5.5 kg in about 84 days with a specific consumption of 2.75 and a breast percentage of 16% of live weight (Grimaud, 2008). Body weight at 51 days is 3.3 kg with a specific consumption of 2.0 kg feed consumption/kg gain.

Female's growth rate has been much smaller compared to male's growth rate and so females are reaching 2.4-3.0 kg at 68 days of age. Egg production has been of 250/female in 46 weeks and fertility has been of over 90%. Hatching period has been of 35 days compared to 28 days for other species/races.

During last 42 years there has been registered a fast genetic advance at this specie. According to researches carried out by Guy (2013), average body weight at slaughtering has been about 5.5 kg; breast weight with skin has been on average 1007 g (18.3%); thigh + drumstick 803 g (14.6%) and abdominal fat 83 g (1.5%).

Researches about feed requirements have shown that this specie does not need rations with high energy level as there has been no growth rate difference until 10 weeks of age when diets in which ME varied between 10.4 and 13.3 MJ/kg have been used (Leclerq and Carville, 1986). Same study has shown that protein requirements decreased quickly from 21% (between 0 and 3 weeks) to 15% (between 6 and 10 weeks). Although this research was carried out more than 30 years ago general observation is still valid.

Grimaud (2008) has shown that Muskovy ducks and the mallard grown for meat have a smaller fat percentage and a higher percentage of valuable carcass parts compared with Muscovy duck.

MATERIALS AND METHODS

This study was performed at Educational Farm Belciugatele - Waterfowl Farm which is located in Moara Domnească and it is belonging to University of Agronomic Sciences and Veterinary Medicine of Bucharest and it is specialized in waterfowl production and breeding and in hatching eggs originated from an original biological material comprising a collection of 4 duck races and 3 goose races for the selection and preservation of sole genetic de waterfowl fund in Romania.

The farm is having two computerized waterfowl houses with an area of 250 square meters each divided in wire pens to keep flocks by races and types and it is also having its own hatching house.

Adult birds body weight and individual egg production has been registered sand eggs were weighted and then they were incubated and incubation parameters have been establishing for the productive evaluation of populations of spotted, black and white Muscovy duck.

Hatching eggs were collected from nests daily usually in the morning when most waterfowl are laying eggs. Eggs are stored inside a room with relative humidity of 60-70% and at a temperature of 12-16°C. Usually ducklings are hatching from the 28th day. Temperature and humidity are very important parameters of which hatching success is dependent. If values are too low or too high ducklings might have deformities or embryo might die. Eggs need a temperature of 37.5°C and an average humidity of 60-70%. Egg candling (ovoscope egg testing) has been carried out from day seven. In last 3 hatching days eggs have been transferred from setter in special hatching crates. Newly hatched ducklings have been kept some hours in the hatchery for to dry themselves and to gain strength.

Some of the hatched ducklings have been kept to replace the parent flock and the others have been sold.

Production and breeding figures have been monitored from December 2019 and yearly average results have been compared with those registered in year 2001 also in Educational Farm of the University. These parameters (monitored weekly / monthly and as average by lying cycle) are the followings: body weight of adult birds (monthly - between December, 2019 - May, 2020), egg production/lying cycle (February - May 2020), egg and chick weight (February - May 2020), fertility and hatching (February - May 2020).

Results were statistically processed by classical means by calculating the average, variation, standard deviation, error of average and variability coefficient.

Student test has been used to study homogeneity of averages and to test the statistical significance of differences observed between averages (between groups) (Sandu, 1995). Calculated Student test value has been compared with its critical (tabular) value at corresponding liberty degrees (cumulated liberty degrees $n_1 + n_2 - 2$) and desired significance level ($\alpha = 0.05$; $\alpha = 0.01$; $\alpha = 0.001$; at a probability of 95%, 99% and respectively 99.99%).

RESULTS AND DISCUSSIONS

Results obtained from the study are presented in the followings for the three lines of Muscovy duck:

At Barbarie duck spotted variety (body feathering is black with white) production and breeding performances of analyzed population are as following:

- females average body weight is 2427 g and males average body weight is 4566 g with no significant differences between months and years;
- birds are having red meat with a low-fat percentage (2% abdominal fat) being also appreciated as "lean duck". Sexual dimorphism is well developed. Late sexual maturity: 26-28 weeks in females and 28-30 weeks in males;
- egg production is 59.42 eggs/bird and egg color is white yellowish and egg weight is 78.21 g with no significant differences between months and years;
- good hatchability - 88.48% with significant differences between months February - May, March - May, April - May and significantly distinct between February - March and February - April.
- hatching percentage is low with an average of 39.79% and it is ranging between 19.90 and 50.07% with very significant differences between months March - April and March - May;
- ducklings weight at day one age was 47.10 g with limits between 45.97 and 47.80 g and without significant differences.

Table 1. Body weight of Muscovy duck populations (grams)

Mention		Spotted			White			Black		
		2020	2001	Student 2001-2020	2020	2001	Student 2001-2020	2020	2001	Student 2001-2020
♂	X	**4566**	**4183**	**2.0050**	**4450**	**3923**	**1.4965**	**4750**	**4222**	**2.3410**
	sx	152.53	115.00		200.01	289.57		180.55	134.94	
	CV	7.47	6.15		10.05	16.50		8.50	7.15	
♀	X	**2427**	**2286**	**0.8356**	**2580**	**2240**	**1.5430**	**2262**	**2280**	**0.0981**
	sx	107.61	129.98		128.07	178.72		135.07	124.10	
	CV	9.91	12.71		11.10	17.83		13.35	12.17	

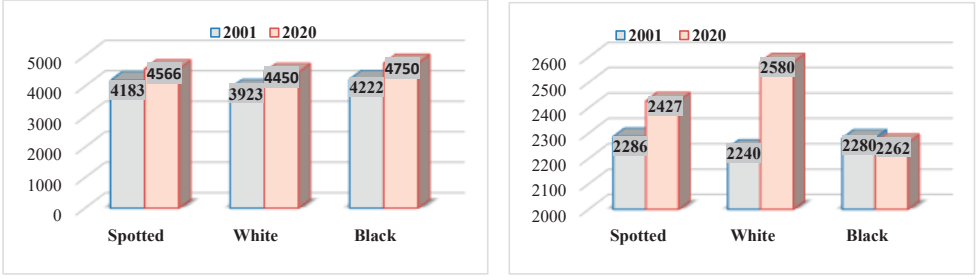

Figure 1. Body weight of males (a) and females (b) of Muscovy duck race (adults, g)

Table 2. Egg production in Muscovy duck

Mention		Spotted		White		Black	
		Total	Student 2001-2020	Total	Student 2001-2020	Total	Student 2001-2020
2020	X	59.42	0.4220	72.06	0.7561	52.45	0.8117
	sx	5.191		8.216		3.064	
	CV	17.47		22.80		11.68	
2001	X	62.52		64.28		55.84	
	sx	5.264		6.193		2.834	
	CV	16.83		19.27		10.17	

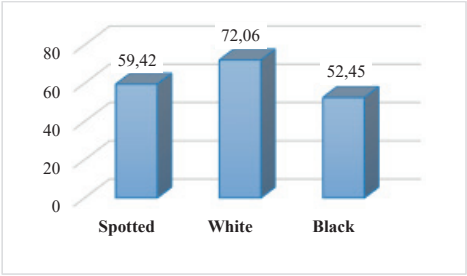

Figure 2. Egg production/lying cycle in Muscovy ducks

The following performances have been registered in white type of Barbary duck (B3, white feathering and orange beak and feet):
- females average body weight is 2580 g (with significant differences between months December, January and May) and males average body weight is 4450 g with significant differences between months December - May;
- sexual dimorphism is sharp;
- sexual maturity is tardy at 26-28 weeks in females and 28-30 weeks in males;
- egg production is 72.06 with eggs of white yellowish color and egg weight of 76.56 g and with no significant differences between months and years;
- fertility is good - 86.00% with significant differences between months February - March and February - April;
- hatching percentage is low - 35.80% ranging between 18.06 and 45.27% with significant differences between years and with very significant differences between months March - April and March - May;
- ducklings body weight at day old age - 48.32 g ranging between 47.18-48.91 g with significant differences between months February - May.

Data processing revealed the following performances in black variety of Muscovy duck (B4, black feathering with greenish metal shine and wings with white spots):
- females average body weight is 2262 g and males average body weight is 4750 g with no significant differences between months and years;
- sexual dimorphism is sharp;
- late sexual maturity: 26-28 weeks in females and 28-30 weeks in males;
- average egg production was 52.45/duck and eggs had a white-yellowish color and a weight of 84.72 g, with significant differences between months February - May;
- fertility is very good - 90.24% with significant differences between months February - April and distinctive significant between February - March;
- hatching percentage - 40.90%, ranging between 20.45 and 51.52% with significant differences between years and with very significant differences between months March - April and March - May;
- ducklings weight at day one age - 49.92 g, ranging between 48.76-51.71 g with significant differences between months May - February.

Table 3. Egg and ducklings' weight in Muscovy duck (grams)

Mention		Spotted			White			Black		
		2020	2001	Student 2001-2020	2020	2001	Student 2001-2020	2020	2001	Student 2001-2020
Egg weight	X	**78.21**	**79.60**	**1.2413**	**76.56**	**78.37**	**1.4930**	**84.72**	**83.52**	**1.0512**
	sx	0.828	0.750		0.765	0.994		0.807	0.805	
	CV	2.37	2.11		2.23	2.69		2.13	2.15	
Ducklings weight	X	**47.10**	**47.97**	**1.2744**	**48.32**	**48.91**	**0.7712**	**49.92**	**48.81**	**1.6891**
	sx	0.499	0.456		0.474	0.583		0.474	0.455	
	CV	2.37	2.13		2.20	2.66		2.12	2.08	

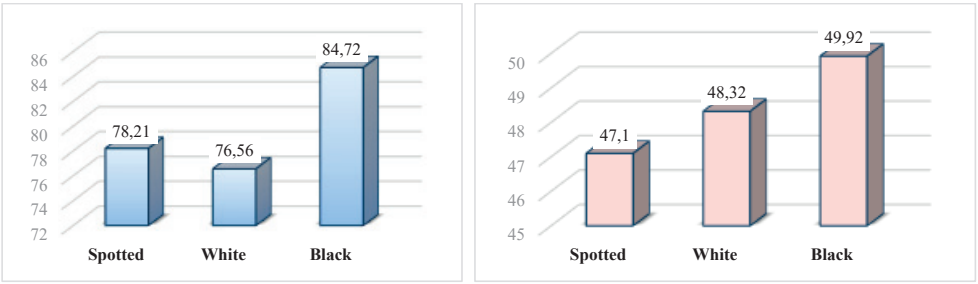

Figure 3. Average egg weight (a) and ducklings' weight (b) in Muscovy duck

Table 4. Fertility (%) and hatchability (%) in Muscovy duck

Mention		Spotted			White			Black		
		2020	2001	Student 2001-2020	2020	2001	Student 2001-2020	2020	2001	Student 2001-2020
Fertility	X	**88.48**	**87.83**	**0.4059**	**86.00**	**82.43**	**2.2208**	**90.24**	**88.11**	**1.3478**
	sx	0.834	1.358		1.229	1.034		0.995	1.230	
	CV	2.13	3.46		3.20	2.81		2.46	3.12	
Hatchability	X	**39.79**	**41.83**	**1.6398**	**35.80**	**41.83**	**4.9105***	**40.90**	**36.65**	**3.4376***
	sx	1.021	0.717		0.998	0.717		0.826	0.918	
	CV	5.74	3.83		6.23	3.83		4.52	5.60	

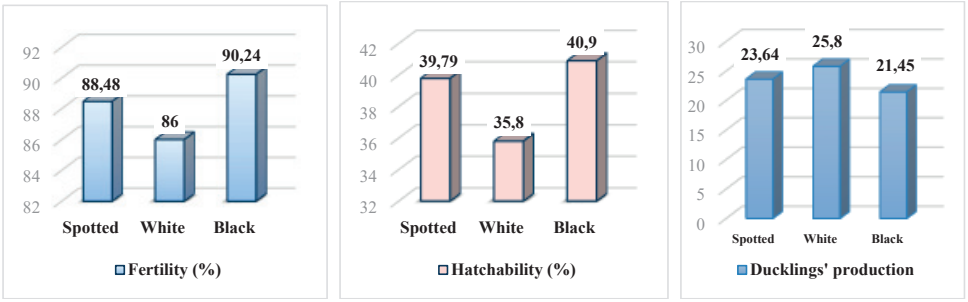

Figure 4. Fertility (%), hatchability (%) and ducklings' production/female in Muscovy duck

It was seen that in Muscovy (Barbary) duck males body weight ranges between 4450 g in line B3 (Muscovy white) and 4750 g in line B4 (Muscovy black) which is used on paternal line in hybridizations and in females body weight ranges between 2262 g in line B4 and 2580 g in line B3. As in the case of males spotted females have an intermediate body weight between the two lines.

Egg production of Muscovy (Barbary) duck ranges between 52.45 and 72.06 eggs/female and line B3 had the best egg production and so this line might be proposed for position of mother in hybridization for the production of mullards parents. Lowest egg production was registered in population B4 with 52.45 eggs (-27.21% compared to B3) smaller than the average of populations followed by the spotted line with 59.42 eggs (3.08% less than the average of all lines).

Average egg weight ranges between 76.56 g in Muscovy white and 84.72 g in Muscovy black.

Ducklings' weight at day one age had the same profile of egg weight curve with one exception in white line of Muscovy duck where egg weight curve is bigger than in spotted line (48.32 g compared to 47.1 g).

Fertility is high in all duck populations monitored. It has been noticed that biggest fertility in Muscovy duck was in line B4 (90.24%) and lowest fertility was in line B3 (86.00 %). These figures especially fitted for this race which is well known for its fertility problems.

Hatching percentage for monitored breeding cycle is low and varies between 35.80% in line B3 and 40.90% in line B4.
Registered ducklings' number by breeding female had the following values: 23.64 ducklings by female were in spotted line and 25.80 ducklings by female were in B3 line and 21.45 ducklings by female were in B4 line.

CONCLUSIONS

The three Muscovy duck lines present in Educational Farm in Moara Domnească (spotted, white and black) had production and breeding performances similar to those described in literature. It should be noticed that they are being the only strains existing in our country and a valuable gene pool which might be the foundation of both obtaining biological material suitable to be marketed and producing mullards by crossing with Peking race of ducks.

ACKNOWLEDGEMENTS

This study was carried out with the support of the Ministry of Agriculture and Rural Development and it was financed inside the frame of project ADER 823/2019.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

WASTE MANAGEMENT IN DAIRY FARMS DURING THE COVID-19 PERIOD - THE CASE OF GJILAN REGION - KOSOVO

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Abstract

The purpose of this paper is to analyze the level of education and experience in animal husbandry in terms of waste management on dairy farms in Kosovo during the Covid-19, the case of Gjilan region. It is a descriptive and quantitative study. Random samples were taken in 71 dairy farms in three municipalities of the Gjilan region. Surveys include farms where 5-78 dairy cows are raised. Data on milk production, waste management from detergents, organic manure, cleaning rags, farm certification and water analysis are included. Datas for each farm were recorded during the period of February-April 2020. During these period farmers' reported that the restrictive measures taken as a result of Covid-19 did not have any negative impact on milk production, however, 26.8% of farmers interviewed reported that milk production was reduced. Further, it was observed a major mismanagement of farm waste that was the main focus of the research: 80.3% of farmers stated that compost waste comes out of the farm and is distributed freely in the environment around the farm.

Key words: *Covid-19, dairy producers, detergent, Gjilan region, manure.*

INTRODUCTION

Agriculture and rural development continue to play a crucial role in Kosovo's economy, as mentioned in the Green Report (2019), "the sector of great importance in the overall economic development of the country". Kosovo continues to be a predominantly rural economy with 7.2 percent of GDP generated by agriculture, during 2018. However, in the last two years (2017-2018) agricultural production has decreased (MAFRD, 2019). Moreover, agriculture is the largest sector of employers, accounting for about 35 percent of the active labour force. (MAFRD, 2019).

In 2018, livestock production decreased by 8.7%, compared to a year earlier, however livestock production is very important for the economic development of the country, as it is one of the most important sub-sectors in agriculture preducing 98% of milk and 60.4% of meat (Krasniqi, 2019).

Cattle is the most important category within livestock, while cows make up 51% of the total of cattle structure. Cow's milk predominates in the production of raw milk - low milk production that does not freeze is insignificant in Kosovo. About 132,500 dairy cows produce 277,599 tons of milk (MAFRD, 2019).

Milk production is considered as an activity of considerable nutritional, social and economic importance in Kosovo. Therefore, the Kosovo Ministry of Forestry and Rural Development (MAFRD) considers the dairy sector as a priority one, providing direct payment supports and investment supports to dairy farmers in order to improve the competitiveness of milk production and improve the standards of food safety and animal health (Zeqiri et al., 2015).

In Kosovo, milk production suffers from a low level of competition, due to low production efficiency and high production costs, and in many cases, producers are forced to accept low incomes by not competing with imported products. (Zeqiri, 2018).

In Kosovo most dairy farmers still haven't solved the issue of waste management such as manure, farm detergent chemicals and other waste at the time of the Covid-19 pandemic. So, we undertook a regional level research in order to see how farms manage these waste issue, knowing that waste mismanagement

directly affects animals and indirectly the human health.

As mentioned by FAO (2020) and Ceylan (2020), COVID-19 has had an impact in many sectors globally and nationally, including the livestock sector. This contagious disease will cause changes in global economy and politics. Traffic restrictions have resulted in difficulties in transporting live animals and animal products such as milk. These restrictions have also caused limited capacity to purchase the necessary production inputs. In many countries, these difficulties have led to a reduction in processing capacity for animal products, as well as to a loss of sales and a slowdown in market activity.

According to Gürel and Yilmaz (2020), in Turkey, livestock and agricultural activities, especially livestock production on an industrial scale, are seen as one of the main sources of natural environment pollution.

Manure management depends on many factors such as herd size and manure type, workforce, soil type, climate and region (Mac-Safley et al. 2011; Smith & Williams, 2016). Moreover, intensive animal production can be significantly problematic in relation to the storage and disposal of manure (Malomo et al., 2018).

FAOSTAT (2020): The amount of wet manure (from animals) can be a major problem for farms. If liquid manure is not used properly, it can create a risk of pollution with a potentially devastating impact on the environment. Improperly deposited manure can flow directly or indirectly into surface waters, as a result, gas emissions and odors can also be released after decomposition of manure, with negative consequences for farmers and ranchers (Font-Palma, 2019). Fangueiro (2008) reported that greenhouse gas emissions (NH_3, N_2O, CH_4), during storage, depend on the type of fertilizer, i.e. emissions from separated solids are usually higher than from liquid or indivisible manure.

Livestock manure contains a wide range of microorganisms that can be a source of risks to human and animal health. These microorganisms can cause food pollution and epidemics and are dangerous to public health (Manyi-Loh, 2016; Malomo et al., 2018).

Therefore, sustainable fertilizer management systems should minimize the environmental risks associated with the storage, treatment and use of manure.

But in recent years, environmental pollution caused by nitrates has been observed, while it is a result of irrational use of natural fertilizers in agriculture (Hokeem et al., 2016). Fertilizers are applied to the soil at a time (usually spreading in the field), so, compared to chemical fertilizers, more leakage occurs and the N content can reach groundwater and surface water (Webb et al., 2010).

As quoted by Hubbard and Lowrance (1989), in the US, there are government regulations that have been imposed on a number of states to protect surface water and groundwater quality from the negative impact of dairy producers, namely dairy cow manure. These regulations may specify the size of land use areas required in relation to the number of cows and may also require monitoring of wells.

But according to FoodPrint (2020), animal manure, unlike human waste, is not treated before it is disposed of. Untreated manure releases chemicals and gases into the air, and when leakage occurs, dangerous pollutants enter our waterways. While human waste is treated in municipal sewage systems and is subject to strict regulation, animal waste are stored in open ponds (called as lagoons) or pits and are used as fertilizer to farm fields. The mixture in the lagoon consists not only of animal feces, but also of bed waste, antibiotic residues, and cleaning solutions as well as other chemicals. Most lagoons are clad only in clay and can flow, allowing wastes to seep into groundwater.

Ammonia, methane and nitrous oxide are prominent pollution concerns in current livestock production (Neumeier & Mitloehner, 2013).

Kosovo promotes and supports legal and natural persons that implement the certified environmental management systems ISO 9000, ISO 14001 and EMAS. Legal and natural persons, who implement certified systems for environmental management, are provided with procedural facilities in the EIA process and in obtaining the environmental permit. (LAW No. 03/L-025, 2009).

European Council Directive 86/278/EEC (1986) on the protection of the environment, and in particular of land where sewage sludge is used in agriculture (the link is external)

regulates the use of sewage sludge in agriculture for prevent harmful effects on soil, vegetation, animals and humans.
Today, many farmers do not have the information to improve fertilizer management or have faced institutional, technical, and socio-economic constraints that prevent them from adopting new practices (CCAC, 2015).
Livestock production is important for food security, food and landscape maintenance, but it also has some environmental impacts. Trans-parental and robust indicators, such as those provided by life-cycle assessment, are required to assess the risks and benefits of livestock production (Leip et al., 2019).

MATERIALS AND METHODS

The study was conducted in three municipalities of the Gjilan region (Gjilan, Kamenica and Viti), Kosovo. Datas were collected during February-April 2020. A structured questionnaire was used to collect all the information related to milk production during the quarter of 2020 consisting to period of the Covid-19 pandemic and the same quarter of 2019. Further, questionnaire was used to collect also information on the farm waste management as fertilizer, use of detergents as chemicals for cleaning equipment that had contact with milk, etc. To avoid confusing questions and to assure clarity, the questionnaire was pre-tested with 6 farmers. Datas from pilot farmers' groups for pre-testing the questionnaire could not be used as Covid-19 restriction measures did not allow collection. 71 face-to-face interviews were conducted, while farms were randomly selected (from the list of farmers of the Agricultural Offices of the Gjilan/Gnjilane Region). Farmers bred 5-78 cows on each of the interviewed farms. The interviewers did not encounter any major problems in terms of willingness to participate, especially after the participants were informed about the purpose of the interview and the survey.
The questionnaire was created to collect information about the general characteristics of the farm, the number of dairy cows, milk yield at different farm sizes; age, educational level of the head of the household, experience as well as the size of the family. Fertilizer waste management, detergents, water analysis from farms are also collected for the period of February - April 2020.
The obtained data were stored in Excel-2000 and imported into SPSS 22.0 for analysis. The stored data were tabulated and adjusted as a percentage value. Descriptive statistics (i.e., frequencies, etc.) were made to estimate the various variables.

RESULTS AND DISCUSSIONS

Socio-demographic indicators of farmers

During this period, the situation of the dairy sector was analyzed with a focus on the relationship between the level of education, experience and practices of farmers for milk production, waste management such as: manure, detergents, water analysis and certification in the three municipalities of the Gjilan region (Gjilan, Viti and Kamenica).
For this purpose, the level of education is included using the Liquid scale of six points: 0 - education or no education, 1 - compulsory education (up to 9 years of school), 2 - agricultural high school (12 years of school), 3 - school other high school (12 years of school), 4 - university degree in environment, 5 - university degree.
The results of group 0-1 (primary education) were compared with those of the group of farmers with better education 2-5 (secondary education +).
Most respondents belong to the age group 30-49 years (56.3%) and 16.9% are over 60 years old. For the group of primary education 66.7% belongs to the age group 30-49 years and for the group of secondary education 55.4%.

Table 1. Main sample socio-demographic and farm indicators

Education Level	Sample farm household indicators				Number of cows			
	Age		Working experience (years)		2019		2020	
	Mean	Stand. Dev.	Mean	Stand. Dev.	Mean	Stand. Dev.	Mean	Stand. Dev.
Primary	49.2	11.55	19.8	13.55	14.53	16.34	14.4	18.31
Secondary	45.3	11.14	15.09	11.27	13.55	9.16	12.48	7.66
Average	**46.1**	**11.34**	**16.08**	**11.81**	**13.76**	**10.93**	**12.89**	**10.67**

Most respondents had less than 20 years of experience in agriculture. The agricultural experience of 53.3 & of respondents was less than 20 years for the primary education group; while in the + secondary education group, 71.4

percent of farmers had less than 20 years of experience. In the group of the most educated farmers 28.6% had less than 10 years of experience. All farms surveyed had more than 4 cows, so they were market oriented. We have targeted market-oriented farms because they are usually more aware of "new situations" and market distortions problems and compared to small subsistence farms (1-2 cows) are more likely to "survive" competition in future growth.

Table 2. Education level of the observed farmers

	Education								
	0[1]	1[2]	2[3]	3[4]	4[5]	5[6]	Total	Mean	Std. Dev.
Total	5	10	10	40	0	6	71	2.54	1.217
%	7.0	14.1	14.1	56.3	0	8.5	100		

The majority (56.3%) of the interviewed farmers have a secondary school level and only 8.5% have a university degree.

Milk production

Milk production for the period of February - April 2020 when compared to the same period of 2019 has increased by 6.7%.

Table 3. Milk production for the period of February-April (2019-2020

Farms	February- April			
	2019		2020	
	Milk production (litre)	Standard deviation	Milk production (litre)	Standard deviation
71	13118	8967.762	13998	9276.314
Minimum	2330		2430	
Maximum	47830		52800	

However, 25 farms (35.2%) report a decrease in milk production by 12.7%. The main reason for the decrease in milk production is the inability to buy food for the cattle, especially during the closure period in the Covid pandemic 19.

Table 4. Daily milk production - Paired sample analyse

Pair	Paired Differences					T	Df	Sig. (2-tailed)
	Mean	Std. Dev.	Std. Error Mean	95% Confidence Interval of the Difference				
				Lower	Upper			
Milk 2020	4.8169	46.1935	5.4822	-61169	15.7505	0.879	70	0.383

[1] No Education
[2] Nine years of education
[3] High agricultural school
[4] High School
[5] Agricultural University
[6] University

The results created for the effect of education level on milk production show that there is no significant difference between groups with different levels of education and milk production in 2019 and 2020.

Table 5. The effect of education level and milk production

Description		Education	Milk 2020	Milk 2019	Education merged
Education	Pearson Correlation	1	-.023	.020	.945**
	Sig. (2-tailed)		.849	.866	.000
	N	71	71	71	71
Milk 2020	Pearson Correlation	-.023	1	.913	.032
	Sig. (2-tailed)	.859		.000	.791
	N	71	71	71	71
Milk 2019	Pearson Correlation	.020	.913	1	.063
	Sig. (2-tailed)	.866	.000		.602
	N	71	71	71	71
Education merged[7]	Pearson Correlation	.945**	.032	.063	1
	Sig. (2-tailed)	.000	.791	.602	
	N	71	71	71	71

Table 6. Use of detergents for cleaning milking machines and milk containers (Descriptive Statistics)

Use of detergents (chemicals)	N	Minimum	Maximum	Mean	Std. Deviation
Detergent for cleaning milking machines and dishes in contact with milk	71	1.0	2.0	1.254	.4381

Table 6 combines the variables on the education levels and experience, in order to see the degree of detergent use with descriptive statistics and we see that out of 71 farms in the research most of them have used detergent minimum once and maximum twice a day.

Table 7. Detergents for cleaning dishes and other milk equipment (Frequency Distribution)

Answering Options		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	**1.0**	53	73.6	74.6	74.6
	2.0	18	25.0	25.4	100.0
	Total	71	98.6	100.0	
Missing	System	1	1.4		
Total		72	100.0		

In Table 7 with the same variables on education levels and experience but with the distribution of statistical frequencies we see that 74.6% of farmers have used detergents for cleaning milking machines and dishes which

[7] Education merged has been recalculated to measure the level of education by degree. Respondents are grouped in three categories: (i): without education + primary education (compulsory), (ii) general high scool;

had contact with milk, while 25.4% have not used at all. To research manure management farmers were asked how they managed manure waste, with many question options being dominated by the answer that manure waste comes out free and is distributed in the yard. From this answer we have derived the essential results of our research see Table below no.8 and see that the majority or (80.3%) of farmers have declared that organic fertilizer waste comes out of the farm and is distributed freely in the environment around the farm (option 2).

Table 8. The effect of education and experience on manure management

Answering Options		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	1.0	8	11.1	11.3	11.3
	2.0	57	79.2	80.3	91.5
	3.0	4	5.6	5.6	97.2
	4.0	2	2.8	2.8	100.0
	Total	71	98.6	100.0	
Missing	System	1	1.4		
Total		72	100.0		

Table 9. Joint education in relation to the use of detergents through the cross table

		Detergent – Machine		Total
		1.0	2.0	
Joint education	0	5	0	**5**
	1	5	5	**10**
	2	38	12	**50**
	3	5	1	**6**
Total		**53**	**18**	**71**

We have also shown the choices that farmers have made regarding the use of detergents through crosstabs (table above), and it is noticeable that farmers with primary education, half of them use detergent, while half of them do not use it at all, those with zero education all use, while of those with high school 38 use, while 12 do not use. To see if the differences are random, we use the Chi Square test, and see that the differences are random (not significant), a result which can be affected by the sample size.

Table 10. Chi-Square Tests

	Value	Df	Asymp. Sig. (2-sided)
Pearson Chi-Square	5.196[a]	3	0.158
Likelihood Ratio	6.019	3	0.111
Linear-by-Linear Association	0.032	1	0.858
N of Valid Cases	71		

a. 5 cells (62.5%) have expected count less than 5. The minimum expected count is 1.27.

Table 11. Group Statistics

	DetMak	N	Mean	Std. Deviation	Std. Error Mean
Exp cow	1.0	52	17.56	12.314	1.708
	2.0	18	11.83	9.691	2.284

From the Table above we see that farmers who use detergents, on average have more experience (average 17.5 years), while those who do not use have less experience (11.8 years). To see if these differences are significant, we used the Independent Sample T test, from which we saw that the difference is nonsignificant, which is most likely influenced by the sample size.

Table 12. Independent Samples Test

		t-test for Equality of Means						
		T	Df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
							Lower	Upper
Exp cow	Equal variances assumed	1.787	68	0.078	5.724	3.203	-.667	12.116
	Equal variances not assumed	2.007	37.416	0.052	5.724	2.852	-.052	11.501

Level of Education and Experience is compared to the question: How far from the farm is the well you get drinking water for households and where you throw waste like cleaning cloths, their detergents after cleaning the cow's breast, car and other equipment that have contact with milk? Analyzed variables: educations levles merged, Farm experience in breeding dairy cows, distance from the farm used for drinking water by farmers and waste disposal. In the following two Tables we see that on average the well from which farmers get drinking water for the family is about 186 meters away from their farm. While from Frequency Distribution we see that the majority of farmers (40.8%) throw waste in the sewer, followed by 23.9% who said they throw it in the stable, and 21.1% who said they throw it in the yard.

Table 13. Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
Wells per farm	71	5.0	5000.0	185.930	612.0533

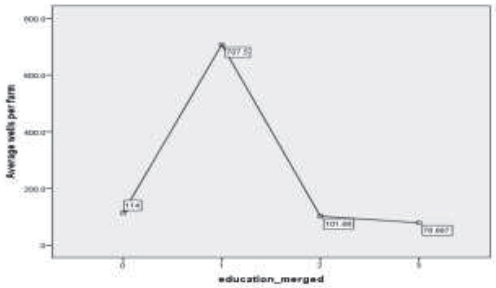

Figure 1. Education in relation to the proximity of the well from the farm

From the Figure above we see that farmers who have completed primary school, have the well on average about 707 meters away from their farm, while it seems that the higher the education, the closer the well. From the table below we see that through the ANOVA analysis we have managed to see that there is a significant difference, at a rate of 95% (sig. = 0.034) between farmers with different levels of education and the distance from where they receive water per family.

Table 14. The distance of the well from the farm

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	3170157.935	3	1056719.312	3.071	.034
Within Groups	23052486.713	67	344066.966		
Total	26222644.648	70			

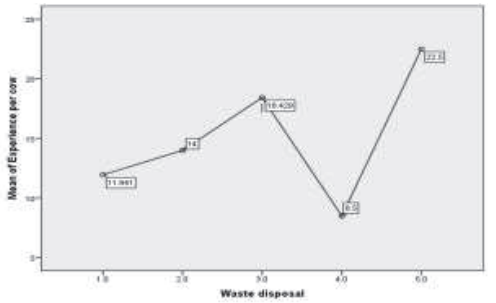

Figure 2. Experience in relation to waste disposal

With waste management there seem to be differences between farmers with different experience levels. Farmers who dump waste in the river (option 4) from the figure above seem to have on average less experience (8.5 years), while those who dump it in the sewer (option 3) or special pits (option 5) have more experience (18.4 years and 22.5 years). To see if the farms are Certified and if they eventually do water analyzes like the chemical and microbiological ones the level of Education and experience has been tested.

Table 15. Certification of the farms

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	4.0	71	98.6	100.0	100.0
Missing	System	1	1.4		
Total		72	100.0		

When asked if the farms are certified, all farmers answered that their farms are not certified to any standard (option 4). When asked if they do water analysis, 93% (66 out of 71 farmers) stated that they do not do water analysis.

Table 16. Water analyses

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	**1.0**	5	6.9	7.0	7.0
	2.0	66	91.7	93.0	100.0
	Total	71	98.6	100.0	
Missing	**System**	1	1.4		
Total		**72**	**100.0**		

CONCLUSIONS

This is an exploratory study, which aims to assess the level of awareness of dairy producers about farm waste management such as stable manure, detergents, rags, water analysis and farm certifications. On the other hand, it also aims to assess the level of the knowledge that they have for the protection of the environment and the side effects that may be caused by waste in public health.

Furthermore, the relationship between the variables of the level of education of farmers and the work experience of farmers was noticed. The sample includes only 3 municipalities in the Gjilan region, due to restrictive measures during the COVID-19 period and financial constraints, however, the findings can be considered indicative of Kosovo as a whole, as the blockade was nationwide .

Statistical analyzes show a high degree of poor waste management as 80.3% of farmers leave manure waste free to leave the farm and they are distributed in the environment around the farm (option 2).

The half of farmers with primary education use detergent, while the other half do not. 38% of

those with high school use it, while 12% do not use. Farmers who use detergent, on average have more experience (17.5 years of experience in avarage), while those who do not use have less experience (11.8 years in avarage). It shows that experience had influenced the use of detergents more than the education had. Thus, it is to be said that knowledge that farmers have gained from non-formal education such as trainings, advices and cooperations with each other had influenced more.

Some farms (38.02%) take drinking water from wells at a distance of 5 to 30 meters away from the farm, which poses a permanent risk of water contamination with bacteria such as *Escheria coli*, etc., thus it is recommended that in these farms water analysis with special emphasis on microbiological ones should be made.

The majority of farmers (40.8%) throw waste from detergents and cleaning cloths in the sewer, followed by 23.9% who stated that they throw them in stables, and 21.1% who stated that they throw them in the yard, without knowing they cause environmental side effects.

It seems that farmers dumping waste into the river (option 4 from Figure; 2 Experience in relation to waste disposal) have less experience in average (8.5 years), while those who dump it in the sewer (option 3) or special pits (option 5) have more experience (18.4 years and 22.5 years respectively)

All farmers responded that their farms are not certified with any standard (option 4), where neither education nor experience had any effect in these terms. Thus, this segment should be addressed by decision-making bodies, due to the fact that such uncertified farms cannot be competitive in international markets.

When asked if they do water analysis, 93% (66 out of 71 farmers) stated that they do not do water analysis at all, which is very worrying for the fact that they are not aware of what water they consume, as well as the potential consequences in case of consuming unhealthy water.

Local and central inspection to implement applicable laws on environmental protection.

MAFRD through ADA to increase funds for the establishment of new farms which aim treating the waste, certification and doing chemical, microbiological and other water analysis in order to protect the health of human and animals.

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TECHNOLOGIES OF THE AGRO FOOD PRODUCTS PROCESSING

Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

MICROSCOPICAL TECHNIQUES USED IN MELISSOPALINOLOGY FOR BOTANICAL ORIGIN OF HONEY DETERMINATION

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Abstract

The paper aimed to present the microscopic techniques used in botanical origin determination of honey samples, shortly melissopalynology. This technique is used for microscopic examination of pollen grains to determine the botanical origin of honey. It is known that a certain number of pollen grains must be recovered from sediment of honey solution, and the presence of small amounts of pollen may be related to falsification. Identification of pollen structure is generally made using light microscopy; phase contrast microscopy may be also used. Fluorescence microscopy is also a powerful method in palynology. Confocal laser scanning microscopy is effective in revealing the ultrastructure of pollen outer layer and shape of the pollen. The determination of the botanical origin of honey using palynology is based on the relative frequency of the pollen belonging to nectariferous plants. Honey is considered monofloral if the pollen from the sediment comes predominantly from a named botanical origin and overpasses 45% from the total count of pollen grains counted on the microscopic slide.

Key words*: confocal microscopy, electron microscopy, light microscopy, palynology, pollen.*

INTRODUCTION

Honey represents one of the most used sweetening agents (Sakač et al., 2019). Honeybees produce this substance that exhibits a great impact for human health and beyond (Abdiniyazova et al., 2016; Nguyen et al., 2018). Nectar of blossoms, secretions of living plants, excretions of plant-sucking insects are some examples of substances that are collected by bees. These species have the ability to combine these substances with their own in order to produce honey (Simsek et al., 2012). The main compounds of honey consist in glucose and fructose, but amino acids, phenolic compounds, organic acids, vitamins, minerals, lipids, enzymes and other phytochemicals are also present in a smaller amount (Baltrušaityte et al., 2007). They depend on many factors such as plant species, climate and environmental conditions, respectively beekeeping practice (Silva et al., 2009). In order to determine the composition and geographical origin of honey, several analytical techniques and parameters combined with statistical methods are needed (Council Directive 2001/110/EC). Honey can be characterised by the aggregation state (liquid), color (light and dark) and can be classified as honeybee (*Apis mellifera*) and stingless bee (*Melipolini*) (da Silva et al., 2013). The produced amount and taste are the major difference between honeybee and stingless bee (Aziz et al., 2017). Different honey properties are related to its composition, especially the minor compounds and residual pollen. These aspects depend on the nectar and pollen of the original plants. Bee pollen possesses antioxidant, anti-inflammatory, antimicrobial, immunomodulatory, antioxidant, antiatherosclerotic, antianaemia, antiallergic, antiosteoporosis and anti-prostatic effects demonstrated by several studies (Gomes et al., 2010; Akbulut et al., 2009; Theunissen et al., 2001). Also, honey is an essential source of polyphenols, flavonoids, sugars, proteins, amino acids, fatty acids, minerals and vitamins (Szczesna, 2006). This bee product can be used as functional food or as nutritional supplement and has an important role in determining the botanical origin.

Microscopic analysis of honey can be done because of the fact that it contains pollen particles, which are concentrated by

centrifugation of diluted honey. Melissopalynology is the main analysis that is used to detect the presence of pollen grains. Also, this type of analysis can identify the floral source and the dominant pollen. If there is more than 45% of one kind of pollen, than it is considerated monofloral honey (Escuredo et al., 2012; Soria et al., 2004). Melissopalynology plays a major role in quality control and origin of honey (von der Ohe et al. 2004; Bryant 2018). Pollen can be a great indicator of the local and regional plant and also can provide information about the floral group used by honeybees to produce honey (Russmann, 1998) Vegetation and climate can be essential factors which have influence above the quantity and diversity of pollen in honey.

Quantitative analysis of pollen in the sediment of honey is used to determine the pollen grain frequency and qualitative control in addition to physico-chemical analysis. If 45% of pollen grain is detected, it is considered predominant, an amount of 16-45% is considered frequent, secondary pollen, and respectively an amount of 3-15% or less is considered sporadic, minor pollen. Due to their pollen amounts, honey can be classified as unifloral or multifloral (polyfloral) (Louveaux et al., 1978). In order to evaluate both the geographical and the botanical origin of different honey types, optical microscopic analysis plays a key role. The present paper aimed to describe some of the microscopic techniques used in melissopalynology in order to denominate different botanical honey samples.

METHODS IN MELISSOPALYNOLOGY

Melissopalynology is a microscopic analysis of honey sediment used to detect the botanical and geographical origin of honey, the pollen types, respectively the source of the flowers (Rech & Absy, 2011). It is the first method which provides information about the botanical characterization of honey. This technique has some limitations such as a requirement of previous knowledge of pollen morphology and specialised employee (Cometto et al., 2003). Optical microscopy is the basic analysis for pollen determination, where a microscopic slide is prepared from honey (simple or using acetolysis), following the procedure described by Louvreaux et al. (1978). Identification of pollen types is made counting at least 500 grains from the slide, with the help of optical microscope, using 40x, 60x magnification, with the help of reference slides of known plants and electronic data bases (Layek & Karmakar, 2016; http://pollen.tstebler.ch).

As stated before, optical microscopy technique requires specialized personnel and time for sample preparation. The sediment of different honeys (10 g honey) is very different in respect of pollen number (due to botanical origin of the sample). It can vary between 1,000 and 10,000. Also the number of distinguishable pollen grains is different and can vary greatly. For this reason, a high percent of pollen grains cannot be determined exactly (down to the species level), and only the higher taxon or the family is determined (Vorwohl, 1967).

The morphological difference between the pollen grains can be determined by using contrast or phase contrast microscopy (Hochuli & Feist-Burkhardt, 2004). Electron microscopy is generally used to differentiate the fine structure of pollen grains (Holst et al., 2007). Fluorescence microscopy has a great impact above analysing cell physiology and it is considerate a common tool of modern cell biologists. One of its major physical limitations is the resolution, which is determined by image contrast and the diffraction of light (Hell, 2003). This method is based on absorption and emission of light energy with the aim to separate them. This process is generally achieved by using optical filters (Helmchen & Denk, 2005). Optical microscopy combined with digital video can quickly and efficiently detect thin optical sections. Wide-field microscopy is used to illuminate simultaneously all parts of the image which allows a faster acquisition. It is also used to analyze specimens in real time (Sheppard & Shotton, 1997). The low cost, simplicity and flexibility of the system are the major advantages of this type of microscope. Toward this, it also has disadvantages such as low image resolution and the possibility for shading artefacts.

Confocal microscopy is based on using spatial filtering to generate a focused spot of illumination with the aim to reject the background light from the image (Helmchen &

Denk, 2005). This process can be achieved with the use of a pinhole aperture which ensures that only the lights from a focused point can reach the detector. The main limiting factor is the speed of the laser scan. Excitation wavelengths on commercial confocal system can include 488, 534, 592 and 635 nm, which means that they are suitable for several fluorescent proteins (Drobizhev et al., 2011).

Electron microscopy is also used in the determination of pollen surface texture, since some decades ago (Laere et al., 1969; Dustmann & von der Ohe, 1993). Scanning electron microscopy is not a routine microscopic determination, because is more difficult to count the pollen grains. This technique is more used for identification of taxa, knowing that same plant family may have similar shape and size of pollen grain (Jones & Bryant, 2007).

RESULTS AND DISCUSSIONS

Pollen grains identification and counting present a huge challenge for the analyst. If simple optical microscopy is used, general information regarding the important plant families may be given (Bobiş et al., 2013; Corvicci et al., 2015; Maida & Özkök, 2020). Also, in bee collected pollen and beebread analysis (other two essential bee products), this method is also used (Mărgăoan et al., 2014; Bobiş et al., 2020; Urcan et al., 2021).

Generally, plant families have similar shapes of pollen, and for this reason using optical microscopy in most of the cases only the plant family may be determined. For more accuracy, other microscopic techniques are required.

Confocal scanning microscopy has proved to be effective in showing details of the fine-structure of pollen exine and more detailed information regarding the shape of pollen grains (Salih et al., 1997; Vitha et al., 2009). Confocal scanning microscopy is based on autofluorescence of the pollen grain (Driessen et al., 1989; Mitsumoto et al., 2009; Castro et al., 2010). In these studies, autofluorescence is used for taxonomical discrimination, on the basis of the intensity and the ratio of the blue to red spectra.

Autofluorescence imaging is considered a non-disruptive method, due to the fact that does not requires any treatment of fixation and staining of the sample. This method could be used in combination with other morphological parameters of the pollen grain in order to identify and quantify correctly the number and species of the grains from the sediment.

A comparation between optical microscopy and confocal microscopy is presented in Figure 1, images made in laboratories of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca.

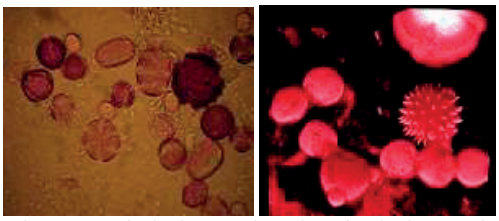

Figure 1. Optical microscopic and confocal microscopic images of multifloral honey sediment (original foto: Mărgăoan Rodica, Tărăban Flaviu)

Using in comparison light microscopy (LM) and scanning electron microscopy (SEM), Jones & Bryant (2007), made a study for morphological comparison of different pollen types and identification of their taxonomy. Although, significantly more taxa were found when using SEM method compared to LM, pollen grains viewed with SEM were divided into three categories: identifiable, obscured and virtually impossible to identify. Taking into consideration the advantages and disadvantages, the authors concluded that there was a minor difference between counting the pollen samples using the two microscopic methods. Every method has its advantages (LM is convenient, SEM have increased resolution of images and more taxa identification), and the final decision for the appropriate method is taken considering the sample, information needed and how much money are available for the study (Jones & Bryant, 2007).

An interesting study (Sivaguru et al., 2012), compared different microscopy techniques used in the analysis of pollen grains. These techniques provide informations on the shape and surface of different pollen types, which present different morphological aspects: widefield, apoptome, confocal, two-photon microscopy, brightfield and differential

interference contrast microscopy and super-resolution microscopy.
The obtained results show that no single optical microscopical techniques capture the pollen shape and the texture of its surface, and only a combination between reflected and transmitted light techniques may recover all morphological information of the pollen, for the exact identification.
A recent study uses three-dimensional (3D) refractive index maps and optical diffraction tomography (ODT) for morphological parameters of the pollen obtained from *Pinus* spp. (Kim et al., 2018).
Figure 2 presents comparatively, original images of different methods of microscopic analysis of pollen grains.

CONCLUSIONS

There appears to be no single optical microscopy technique that can satisfactorily show pollen shape and texture of the pollen surface. For this reason, a combination of reflected and transmitted light techniques is required to maximize the correct identification and quantification of pollen from both the sediment of different honey types, and from bee collected pollen or beebread.
The intraspecies pollen differences can be highlighted using different microscopic techniques and specialized personnel.
Although palynological analysis is apparently an easy determination, a high specialization is needed, both in microscopy and botany.

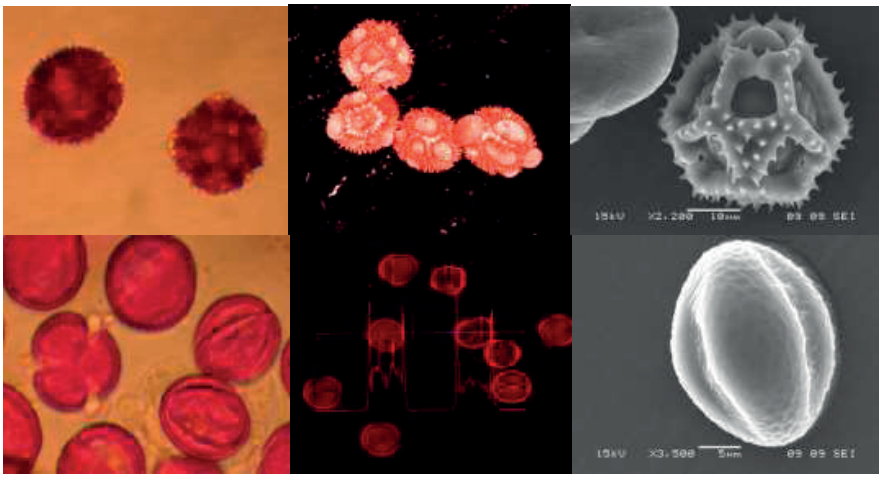

Figure 2. *Taraxacum officinale* and *Salix* spp. pollen optical, confocal and electron microscopic pollen images (original foto: Mărgăoan Rodica, Tărăban Flaviu, Varadi Alina)

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

COMPARATIVE STUDY ON SOME VEGETABLE OILS PRODUCTION TECHNOLOGIES AND THE IMPACT ON THEIR PHYSICO-CHEMICAL INDICES

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Abstract

Vegetable oils are a good source of high quality lipids, ω-3 and ω-6 acids, vitamin E and other biologic active components recommended to be included in a healthy diet. Based on the type of oil seeds and processing technology, the composition and physico-chemical proprieties of oils differs. For the research, we used samples of sunflower oil (pressed and refined), soybean oil, olive oils and nuts oil, which were characterized accordingly to standards, based on certain characteristics (phosphatides level, acidity index, peroxide index, saponification index, refraction index, neutralization index). The results have shown the influence of processing on the final characteristics of oils and also helped to draft and understand the recommendation regarding their storage.

Key words*: fatty acids, oils, physico-chemical characteristics, quality, technology.*

INTRODUCTION

Most vegetable oils are obtained from beans or seeds, which generally furnish two valuable commodities: a fatty oil and a protein-rich meal. Seed extraction is achieved by pressing and/or by extraction with hexane. Oils such as palm and olive, on the other hand, are pressed out of the soft fruit (endosperm).

Some oils, such as virgin olive oil, are used without further treatment other than filtering, but most are refined in some measure before use. The refining processes remove undesirable materials (phospholipids, monoacylglycerols, diacylglycerols, free acids, colour and pigments, oxidised materials, flavour components, trace metals, sulphur compounds and pollutants), but may also remove valuable minor components, including antioxidants and vitamins such as carotenes and tocopherols. The refining processes must therefore be designed to maximise the removal of undesirable components and minimise the removal of the valuable minor components. Some of the latter are recovered from side streams of the refining process to give commercial products such as phospholipids, free acids, tocopherols, carotenes, sterols and squalene. Because of changes that occur during refining, it is important to know whether compositional data refer to crude or refined oil (Gunstone, 2002).

Vegetable oils and fats are also used in the confectionery products; their market is increasing and evolving into a diverse range of products, which are highly appreciated by consumers (Bahaciu, 2019).

Aging represents a great concern in developed countries because of the number of people involved and the pathologies related to it, like atherosclerosis, Parkinson, Alzheimer's disease, vascular dementia, cognitive decline, diabetes and cancer.

The Mediterranean diet, rich in virgin olive oil, improves the major risk factors for cardiovascular disease, such as the lipoprotein profile, blood pressure, glucose metabolism, and antithrombotic profile. Endothelial function, inflammation, and oxidative stress are also positively modulated. Some of these effects are at attributed to minor components of virgin olive oil (Boskou, 2006).

MATERIALS AND METHODS

Materials

We have analyzed five types of vegetable oils: refined sunflower, canola oil, soybean oil, olive oil and nuts oil. The samples were bought from the market, they were packed and ready to consume.

In order to analyze the exposure to oxidation, we have investigated samples of cold pressed filtered sunflower oils stored in cold dry place for 4, 24, 52, 72 and 110 weeks. The samples were obtained from a local producer based in Vrancea county.

Table 1. Codes used for samples analysis

Sample	Code
Refined sunflower oil	RSF
Cold pressed filtered sunflower oil 4 weeks	FSF-4
Cold pressed filtered sunflower oil 24 weeks	FSF-24
Cold pressed filtered sunflower oil 52 weeks	FSF-52
Cold pressed filtered sunflower oil 72 weeks	FSF-72
Cold pressed filtered sunflower oil 110 weeks	FSF-110
Canola oil	Can-O
Soybean oil	Soy-O
Olive oil	Olive-O
Nuts oil	Nut-O

Methods

The official methods used in order to characterize the chemistry of analyzed oils were:

- the acid value (free fatty acids) accordingly with EN ISO 660 standard methodology;
- the saponification value - accordingly with EN ISO 3657;
- the peroxide value - accordingly with EN ISO 27107;
- the iodine value - accordingly with EN ISO 3961;
- the refractive index - accordingly with ISO 6320: 2000.

RESULTS AND DISCUSSIONS

1. Comparison of the main quality parameters of analyzed samples

1.1. The acid value (free fatty acids)

Oils degradation during processing and storage is determined by a range of hydrolysis and oxidation reactions due to oxigen exposure and water content of oils. This has an important effect on oils nutritional and sensorial quality, safety and acceptability.

By enzymatic hydrolysis of triglycerides, free fatty acids level is increasing thus the risk of double bonds oxidation is also increased.

The acid value is determined as the quantity of free fatty acids hydrolyzed by KOH added to titration. It is expresses as % of oil acid.

The higher the acid value is, the higher the free fatty acid in oils.

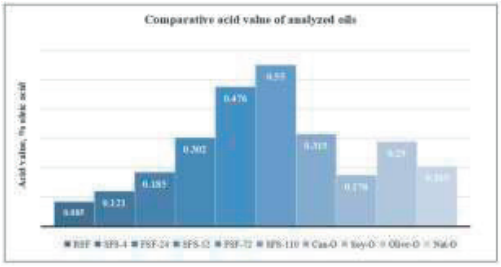

Figure 1. Comparative acid value of analyzed oils

In Figure 1 it is shown that the highest acid value was observed for cold press filtered sunflower oil stored for 110 weeks. This was expected first of all because the cold press filtered sunflower oil (not refined) had an increased water content which facilitated the hydrolysis and secondly, during such a long period of storage time, the lipolysis process is intense and determine the accumulation of free fatty acids in oil.

Among the varieties of oils, the higher acid value was observed for canola oil (0.315% oleic acid) and olive oils (0.29% oleic acid). The smaller acid value was determined for refined sunflower oil (0.085% oleic acid).

1.2. The saponification value

Saponification value represents the number of KOH milligrams required for the saponification of 1g oil. It depends on the molecular mass of the oil. The higher the molecular mass is, there will be less molecules of triglycerides/g, so, the smaller the saponification value will be. High molecular mass lipids will have low saponification value.

In Figure 2 it is represented the comparison among oils regarding the saponification value.

It can be observed that the values are quite similar, with the exception of canola oil which has the saponification value of 181.14 mg KOH/g oil.

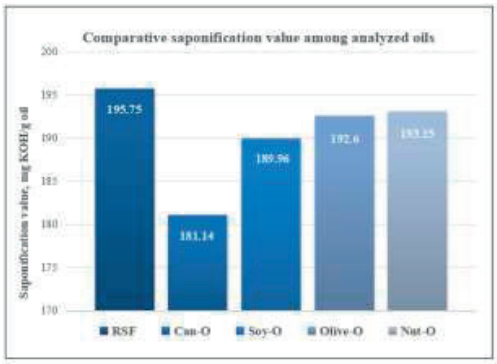

Figure 2. Comparative saponification value among analyzed oils

1.3. The peroxide value

The peroxide value is determined in order to establish the freshness of oils, by showing the incipient phase of oil oxidation, taking into consideration that peroxides are the first components of oil oxidation.

The increasing rate of the peroxide value of an oil depends on the storage conditions (temperature, time, light exposure and humidity) and it is used to evaluate the stability of an oil.

The purpose of peroxide value determination was to establish the oxidative alteration status in analyzed oils. A sensorial analysis was not conducted in this experimental plan, but when preparation of samples was done it was observed an increased rancid scent for samples that were registered the higher peroxide value.

In Figure 3, the comparative peroxide value among analyzed oils are shown.

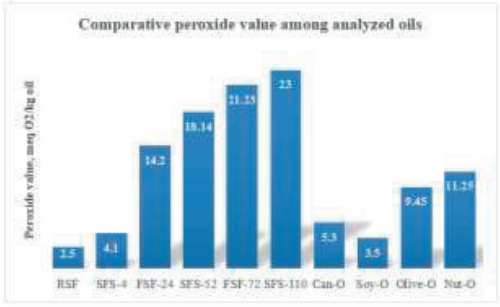

Figure 3. Comparative value among analyzed oils

The highest number for peroxide values were obtained for samples of cold pressed filtered sunflower oils. The more they are stored, the higher the peroxide value rise.

Regarding the other oils, the highest score for peroxide value was observed for nuts oil (11.25 mEq O_2/kg oil), followed by olive oil (9.45 mEq O_2/kg oil). The smaller peroxide value was for refined sunflower oil (2.5 mEq O_2/kg oil).

A detailed analysis on the influence of the storage time on the peroxide value for cold pressed filtered sunflower oil will be presented below, at point 2.

1.4. The iodine value

The iodine value indicates the degree of unsaturation of oils and it represents the amount of Iodine (grams) added to 100g oil. The higher the Iodine value is, the more unsaturated the oil is (more double bonds, more Iodine needed to addition to them, higher Iodine value).

It was expected that the oils with high level of unsaturated fatty acids content to have an increased value of the Iodine value.

In figure 4, it can be observed that nuts oil has the highest Iodine value (153.12 g I_2/100 g oil), followed by canola oil (146.10 g I_2/100 g oil) and refined sunflower oil (141.15 g I_2/100 g oil).

It also can be observed that the Iodine value for stored cold pressed filtered sunflower oils decreased up to 118.15 g I_2/100 g oil after 110 weeks of storage. This can be explained by the decrease of the number of double bonds due to oxidation.

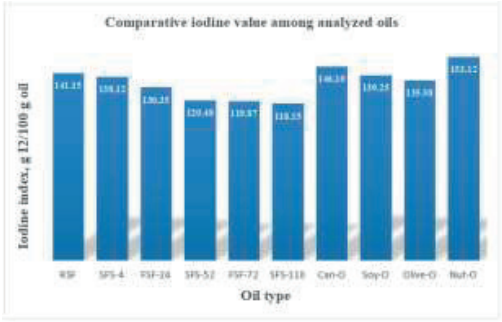

Figure 4. Comparative iodine value among analyzed oils

1.5. The refractive index

The refractive index is a parameter which is correlated with molecular mass, fatty acids constituents, length of their chain, unsaturation

degree and also the degree of conjugation of double bonds.
On the other hand, the refractive index increases with the size of the carbon bond chain in fatty acids and with the number of double bonds present in fatty acids (O'Brien, 2009).
This index is a measure of the angle at which the light falls when refracted by changing direction at the boundary between two media.
For the analyzed oils, the values of the refractive index are shown in Figure 5.

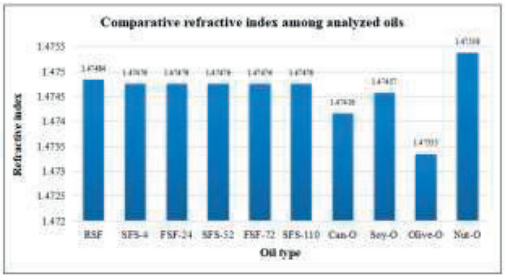

Figure 5. Comparative refractive index among analyzed oils

Although it is not a uniform correlation, it can be seen that nut oil (with a large number of fatty acid double bonds) has the highest refractive index, respectively 1.75375.
The next should be the refractive index of olive and rapeseed oil, but probably due to their high peroxide index, the value of the refractive index was also influenced (Oycan, 2009).

2. Evaluation of exposure to oxidation

Vegetable oils and fats are rich in unsaturated fatty acids, ω-3 and ω-6, which are recommended by nutritionist to be consumed for a good health of the heart and artery.
Unsaturated fatty acids are involved in prostaglandine synthesis, modulation of some essential functions like smooth muscle tone, inflammatory response, renal function, nervous system functions (Segal, 2002).
During storage, due to time, temperature, light exposure, oxidation is initialized and unsaturated fatty acids are involved. This determines changes in sensorial (rancid smell and taste) and chemical characteristics (Gunstone, 2008).
The main parameters that change are acid value (Figure 6) and peroxide value (Figure 7).

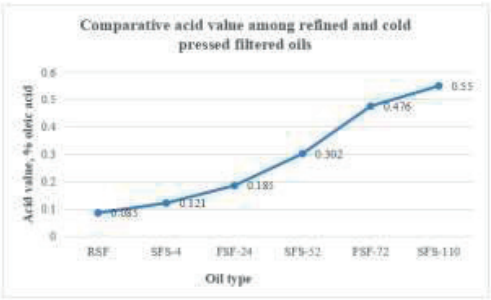

Figure 6. Comparative acid value among refined and cold pressed filtered oils

As regarding the acid value of cold pressed filtered sunflower oil, it can be observed that this was starting to increase in the first 4 weeks of storage and reaches 0.55% oleic acid after 110 weeks of storage, which means 6.47 times higher than refined sunflower oil and 4.55 times higher than cold pressed filtered sunflower oil stored for 4 weeks.
Furthermore, the processing technology, which not include refining, influence the composition of oils: cold pressed oils contain an increased amount of free fatty acids, waxes, odors, colour compounds, enzymes, which can also influence the oxidation process. (Hamilton, 2002).

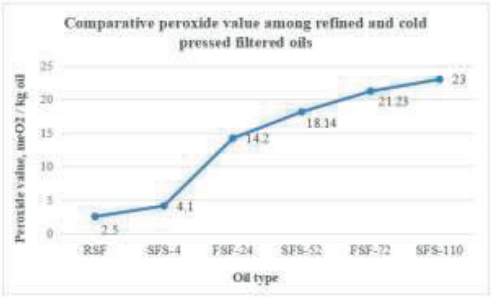

Figure 7. Comparative peroxide value among refined and cold pressed filtered oils

A separate analysis of the four types of sunflower oil, shown in Figure 7, shows that as the oil storage time increases, the peroxide index increases.
It is very clear that by storing sunflower oil for a very long time, the peroxide index increases, reaching values of 23 meq O_2/kg oil.
It is also interesting that, although packaged in brown glass packaging, olive and walnut oils, which are valid, recorded quite high values of peroxide indices: 9.45 and 11.25 meq O_2/kg,

respectively. This can only be explained by the possible storage of these oils at high temperatures.
From the above information, but also from the study of the literature (Gurr, 2009; Nawal-A-Al Badr, 2014), the acidity index and the peroxide index are both indicators of the degradation status of vegetable oils: the acidity index, the hydrolytic and oxidative degradation.
We thought it would be interesting to follow the evolution of these two parameters in the same graph, to see if they correlate.
As a result, in figure 8 it can be observed the correlation of peroxide and acid values for all analyzed oils.

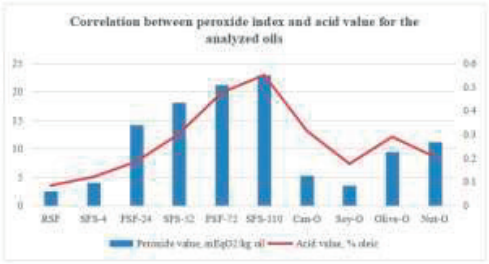

Figure 8. Correlation between peroxide index and acid value for the analyzed oils

Figure 9 shows the acidity and peroxide indices for the sunflower oils analyzed.
We observed what was intuitive: both indices increase with the exposure of sunflower oil to the action of hydrolases or factors that increase the degree of oxidation.

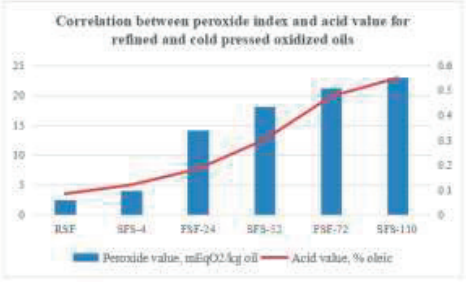

Figure 9. Correlation between peroxide index and acid value for refined and cold pressed filtered oils

CONCLUSIONS

1. Depending on the technological process adopted, the chemical composition of the oils is different.
2. The initial processing method, the oil extraction method (by pressing or extraction, or both) also influences the compositional qualities of the vegetable oils taken into analysis.
3. The type of oil processing, the existence or not of the refining process, influences the nutritional qualities of the oil, on the one hand, but also their stability, on the other hand.
- ✓ In the case of cold-pressed oils, they retain all the vitamins and nutrients from the raw material, but at the same time its stability over time is reduced, mainly due to the high water content.
- ✓ Refined oils have the advantage of obtaining uniform batches, eliminating differences in color, taste and smell between batches, but at the same time the oil may contain traces of chemicals (hexane) and saturated fatty acids (trans fats) (Bahaciu, 2007).

4. The evaluation of some biochemical parameters of essential oils established the dependence of the parameters investigated by each other, but also on the methods of oil manufacture.
5. The use of empirical mathematical formulas can give indicative indications on some indices, but for concrete and correct values laboratory analyzes specified in the specific standards on the product must be performed.
Using antioxidants in oils could improve their stability to peroxidation during cooking and home processing.

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***EN ISO 3961. Iodine value

***EN ISO 27107. Peroxide value

***ISO 3657.Saponification value

***EN ISO 660. Acid value, free fatty acids (FFA).

Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

STUDY ON SOME PHYSICOCHEMICAL PARAMETERS IN GOAT'S MILK AND WHITE BRINED CHEESE IN THREE GOAT BREEDS

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Abstract

The main physicochemical parameters of goat milk of three breeds-Bulgarian White Dairy (BWD) and its crosses with Anglo-Nubian (BWDxAN) and Togenburg (BWDxTG) for lactation period were studied and three batches of white brined cheese produced from the milk breed were prepared. Goat's milk processed into white brined cheese from the studied breeds of goats reared in one herd is characterized by the lowest content of dry matter (DM) - 13.03%, milk fat- 4.91%, solids non fat (SNF) - 8.32%, total protein - 2.97%, casein - 2.03%, calcium - 0.139% and density - 28.2ºG, in BWD breed compared to its crosses. In the cheese at the 24-th hour of production, the water content is the lowest in the milk cheese of the BWD breed-53.34%, and the values for protein and milk fat 15.16%, 24.0% are the highest in the batch produced from the milk of the same breed. In mature white brined cheese on the 45-th day of production, the water and protein content decreased in all three batches compared to the 24-hour cheese, while the values for milk fat, fat content in the dry matter, water in the non-fat residue and salt in the aqueous phase rises.

Key words*: goat, milk, physicochemical composition, white brined cheese.*

INTRODUCTION

In terms of chemical composition and biological properties, milk is one of the most complete products of animal origin, rich in nutrients and biologically active substances. Its composition and properties are closely related to its hygienic, nutritional, technological and organoleptic characteristics, corresponding to the main ingredients (fats, protein, lactose) and some other components contained in milk such as minerals, vitamins, cholesterol, terpenes (Morand-Fehr et al., 2007), as well as diverse cells, including glandular epithelial cells and leukocytes, including macrophages, neutrophils and lymphocytes. As noted by Raynal-Ljutovac et al. (2005), protein and fat are the most important indicators of raw milk, as well as the main technological criteria due to their contribution to cheese yield, taste and other characteristics of dairy products.

Goat's milk is characterized by good digestibility, alkalinity, buffering capacity and certain therapeutic characteristics valuable for medicine and human nutrition (Haenlein, 2007; Park et al., 2007).

Goat's milk is characterized by a large variation in chemical composition, technological and hygienic indicators, depending on various factors - genetic factors, climatic conditions, conditions and method of cultivation and more (Plakantara et al., 2010).

The cheese composition changes depending on the lactation phase and corresponds to the changes in the milk composition. Cheese made from milk obtained at the end of lactation has a higher content of fat, protein and dry matter, which corresponds to higher values of these indicators in milk during this phase of lactation (Soryal et al., 2005). The milk production season also has an impact on the variation of the chemical and coagulation characteristics of milk (Zullo et al., 2005). Even the time of milking during the day affects the milk's potential for cheese production. The milk obtained during the morning milking is characterized by better coagulation qualities, higher coagulation rate and better consistency of the curd (Zullo et al., 2005).

Kondyli et al. (2016) found for white brined goat's milk cheese from the 48th hour of production to the 60th day, respectively, a

slight increase in water content values (54.56-55.47%), milk fat (25.13-25.50%), and fat content in dry matter (55.31-57.30%), while the protein content decreased from 17.45 to 16.01%.

The objective of the present study was to monitor changes in some physicochemical parameters in goat's milk and the resulting white brined cheese on the 24th hour and 45th day of the maturation process, respectively, in three groups of goats Bulgarian White Dairy (BWD) breed. and its crossings with Anglo-Nubian (BWDxAN) and Toggenburg (BWDxTG) for lactation period.

MATERIALS AND METHODS

The experiments were conducted at the Experimental Base at the Research Institute of Mountain Stockbreeding and Agriculture, Troyan. Experimental animals raised in one herd of three groups were used - Bulgarian White Dairy goat breed and its crossings with Toggenburg and Anglo-Nubian. The animals are aged from 3 to 5 years (second-fourth lactation), and the kiddings were in February. The rearing system is stable-pasture, and during the period April-November the animals were on a natural pasture of transitional type and in stable, during the rest of the year.

The milk samples for analysis were taken from morning milking at the beginning, middle and end of the lactation period (April-June-September), for which purpose the animals were milked manually, observing the necessary hygienic conditions. Nine samples of aggregate milk (3 x 3 pieces) during the lactation period of TNMO the three groups of animals were examined to determine the content of dry matter, milk fat, dry fat-free residue (DFR), protein, casein, non-casein protein, lactose, calcium (Ca), density, titratable and active acidity, coagulation, total number of microorganisms (TNMO) and total number of somatic cells (TNSC). The samples were analyzed in the technological laboratory for milk and dairy products at RIMSA - Troyan. Three batches of white brined cheese were prepared from the aggregate samples of goat milk from the three groups of animals at the beginning, middle and end of the lactation period (9 batches in total) under laboratory conditions, according to the methodology described by Peychevski et al. (1988) without standardization of milk. Samples of the obtained batches of cheese were examined on the 24th hour and on the 45th day of the ripening process.

The following milk parameters were tested on the Milko-Scan FT 120 (Foss Electric):

*Chemical indicators:*Dry matter, Milk fat, Dry fat-free residue, Total protein, Casein protein, Lactose, Calcium - complexometric (Kondratenko et al., 1981); Non-casein protein - by calculation;

Physical indicators: Density - with lactodensimeter, BDS 1110-73; Titratable acidity - by the Turner method, BDS 1111-80; Active acidity (pH) - potentiometrically; Coagulability - according to Dimov et al. (1974); Syneresis of the rennet coagulum - by the method of Shidlovskaya (1979), modified by Peychevsi (1983);

Microbiological parameters of milk

The tests for TNMO and TNSC were performed in the *Alimenti* Testing Laboratory at D&V Consult OOD, Tsaratsovo village, Municipality of Plovdiv; Total number of microorganisms (TNMO) - BDS ENISO 4833-1: 2013; Total number of somatic cells (TNSC) - BDS ENISO 13366-1: 2008;

Dry matter, milk fat, total protein, water content, salt of cheese were examined on Food Scan device (Lab, 78800)

Milk fat in dry matter (MFDM), water in the fat-free residue (WNFR), salt in the aqueous phase (SAP) were found by calculation (Lawrence & Gilles, 1980).

Titratable acidity - by the method of Turner, BDS 1111-80;

The variational-statistical data processing was done through Statistica software package, and the graphic - through Excel. The mean values of the groups in the individual studies were compared according to the tables of the Student-Fisher t-test.

RESULTS AND DISCUSSIONS

Differences in the level of milk productivity and milk composition are influenced by genetic and physiological factors such as breed, individual characteristics, lactation phase, animal husbandry, climate, botanical

composition of grassland (Scintu & Piredda, 2007). The goat's milk tested has physico-chemical characteristics specific to the species studied and meets the requirements of Regulation 853/2004 (Table 1).

Table 1. Composition and properties of goat's milk processed into white brined cheese (n = 3)

Indicators	Groups		
	BWD	BWD x TG	BWD x AN
	x ± Sx	x ± Sx	x ± Sx
Dry matter, %	13.03±0.316	13.51±0.443	13.62±0.400
Milk fat, %	4.91±0.409	4.97±0.354	5.19±0.302
Dry fat-free residue, %	8.32±0.085a*	8.76±0.129b*	8.63±0.101
Protein, %	2.97±0.129	3.26±0.081	3.38±0.101
Casein, %	2.03±0.062	2.14±0.061	2.20±0.035
Non-casein, %	0.87±0.058	1.12±0.092	1.18±0.081
Lactose, %	4.37±0.255	4.51±0.196	4.25±0.222
Ca, %	0.139±0.007	0.148±0.003	0.144±0.004
Ratio K/M	0.413±0.023	0.430±0.028	0.424±0.043
Density, $^{o}G_{20/4^{o}C}$	28.2±0.402	29±0.343	28.3±0.365
Titrable acid, ^{o}T	15.17±1.093	15.33±0.726	14.83±0.928
Active acidity, pH	6.48±0.050	6.50±0.047	6.53±0.052
Curdling, s	294±0.120	285±0.128	283±0.145
TNMO cfu/ml	8.40 x 10^5±0.436	6.27 x 10^5±0.467	7.13 x 10^5±0.549
TNSC n/ml	1.90 x 10^5±0.436	1.67 x 10^5±0.418	2.13 x 10^5±0.406

Note: a - BWD; b - BWD/BWD x TG; *P≤0.05

The composition and properties of the cheese depend mainly on the composition and properties of the milk from which it has been produced (Peychevski, 1983). The main ingredients characterizing the cheese as a food product are milk fat, dry fat-free residue and water content, the latter affecting the taste, texture, structure and type of cheese (Chomakov et al., 2000).

The highest results for dry matter were reported in BWD x AN - 13.62% and BWD x TG - 13.51% compared to BWD breed - 13.03%, which is close to the values obtained by Soryal et al. (2005) for Nubian goats - 13.45%, raised in the USA and lower than those of Narangerel et al. (2016) - 15.23% for goats in Mongolia.

Milk fat, dry fat-free residue and protein score high in BWD x AN (5.19%, 8.63%, 3.38%) and BWD x TG (4.97%, 8.76%, 3.26%) compared to BWD breed, which is lower than mean values for DFR and protein - 9.1% and 3.60% found by Dimassi et al. (2006) in goat milk of Dahlem Cashmere breed raised in Germany. It is close to protein values found by Johanson et al. (2015) - 2.93%, 3.20% and 3.39% in Swedish White breed with low, medium and high levels of a_{s1}-casein and close to those obtained by Zullo et al. (2005) for milk fat (4.62%-5.23%) in several herds of Cilentana goats with different pigmentation, raised in the Salerno region. Damian et al., (2008) reported a milk fat content of Saanen and Anglo-Nubian breeds of 3.59% and 4.65%, respectively, which is lower than our results, and Šlyžius et al. (2017) found values for milk fat in Anglo-Nubian goats in Lithuania - 5.20%, which coincides with our results for BWD x AN - 5.19%.

The data on dry matter, milk fat and protein obtained in the present study in all three groups of goats are lower than those of Raynal-Ljutovac et al. (2008), respectively 14.8%, 5.63% and 4.09% and higher than those found by Abbas et al. (2014) for Saanen goat breed in Nigeria - 12.15%, 3.41% and 3.07%.

The variation in the casein values between the three milks was insignificant and was due to the breed and the lactation phase, as the highest results were found in BWD x AN - 2.20%, which coincides with that obtained by Imran et al. (2008) - 2.18% casein in goat's milk tested in Pakistan, and the lowest values were reported in BWD breed - 2.03%. Compared to our results, Peychevski et al. (1986) also found a low casein content (2.08-2.32%) in goat's milk processed into white brined cheese, but

with a significantly lower non-casein protein content (0.65-0.68%), similar to the studies of Albenzio et al. (2006) for the aggregate milk from four goat herds during the spring season - 0.35-0.60% (2.5-2.9% casein).

The amount of non-casein protein varied in a relatively narrow range from 0.87% in BWD to 1.18% in BWD x AN.

Lactose had the lowest results in milk from BWD x AN - 4.25%, and the highest in BWD x TG - 4.51%, which is close to those indicated by Tudisco et al. (2014) - 4.57-4.65% lactose in the milk of goats raised fon pastures and stables in Italy.

Variations in calcium values between groups are minimal and coincide for BWD with those found by Park et al. (2007) - 0.134% calcium in goat's milk, but lower than those obtained by Rawya and Ahmed (2014) - 0.200% in Damascus goats in Cyprus.

As the milk density changes depending on the content of milk fat and dry matter, the lower content of dry matter in the milk of BWD breed, determines the lower density of this milk (28.2ºG) compared to that of BWD x TG (29.0ºG), as the differences are statistically insignificant ($p>0.05$) and coincide with the results obtained by Odzhakova, (2002) for milk density of local goats - 28.6ºG and crossings - 29.1ºG raised in the Middle Rhodope Mountain.

There were no significant differences between the groups in terms of active and titratable acidity, in contrast to the data from some studies abroad - pH 6.36 ÷ 6.82 (Helmut & Fiechter, 2012) and 5.69 ÷ 6.92 (Dračková et al., 2008) and 11.5 ÷ 20.5°T (Dračková et al., 2008).

Mihailova et al. (2000) indicate values for titratable acidity in BWD - 15.8ºT and for local goats - 15.6ºT, which is close to the one obtained in the present study for BWD breed - 15ºT.

Imran et al. (2008) found identical to our results active acidity - 6.59 in goat milk in Pakistan.

Bhosale et al. (2009) found lower data on active acidity from 6.23 to 6.49 in the milk of first to fourth lactation goats in India

Iancu (2010) reported pH values from 6.25 to 6.38 in first to seventh lactation goats in Sibiu (Romania), which confirms our claim that the differences in the values of these indicators are probably due to nutrition, climatic conditions, individual characteristics and health condition of the animals, the specificity of the area, etc.

One of the most important indicators in cheese-making, on which the structure, yield and quality of the cheese largely depends, is the curdling capacity of the milk and the syneresis of the obtained rennet coagulum (Peychevski, 1983).

Coagulation and the conditions of syneresis determine the final characteristics of the cheese, due to their impact on the moisture and protein content. The dry matter content, the composition of the whey and the characteristics of the final product are determined mainly by the control of the syneresis process and the separation of the whey applied during processing into cheese (Garćia et al., 2014).

The milk used has a very good curdling capacity of 283 s for BWD x AN, 285 s for BWD x TG and 294 s for BWD.

Mihailova et al. (2000) found the curdling capacity of goat's milk of BWD breed - 266.4 s, for crossings - 291 s and for domestic goats - 239.2 s. Peychevski et al. (1986) found a much faster curdling of milk from Saanen goats (53.3 ÷ 57 s), probably due to the influence of higher titratable acidity in their study - 17.7 and 18.7°T (Gorbatova, 1984) or the content of $\alpha s1$-casein and the higher degree of dispersion of casein micelles (Vegarud et al., 1999).

In contrast to the present results and those of Peychevski et al. (1986), Clark & Sherbon, (2000) found significantly slower curdling of milk from 6 breeds of goats and some of their crossings - between 346 s and 964 s, 531 s for that of Nubian and 829 s of Toggenburg breed.

According to Regulation (EC) No 853/2004 laying down specific hygiene rules for food of animal origin, raw goat's milk must contain no more than 1,500,000 total number of micro-organisms/ml (TNMO/ml), while the number of somatic cells in goat's and sheep's milk has not set a limiting upper threshold (Jimenez-Granado et al., 2014).

The total number of microorganisms in the milk processed into white brined cheese varied from 6.27 x 105 cfu/ml (BWD x TG) to 8.40 x 105 cfu/ml (BWD).

Carusillo et al. (2014) examined 536 samples of raw goat's milk and found that in 85.1% of

the samples OTNMO was below 1,500,000/ml, as most of them (80.2%) contained TNMO below 500,000/ml.

Cupáková et al. (2012) found an average monthly total number of microorganisms in the order of 4.53-5.21 log cfu/ml, with the highest peak in July.

In the absence of mastitis, the number of somatic cells in goat's milk can vary from 270 x 10^3 to 2000 x 10^3 cfu/ml (Jiménez-Granado et al., 2014).

Processed milk from BWD and BWD x TG contained significantly less TNSC - 1.90 and 1.67 x 10^5 pcs/ml, respectively, compared to that of BWD x AN - 2.13 x 10^5 cfu/ml.

The speed and amount of whey released from the milk coagulation is an important technological indicator. The studied goat's milk had a well-defined syneresis (Figure 1).

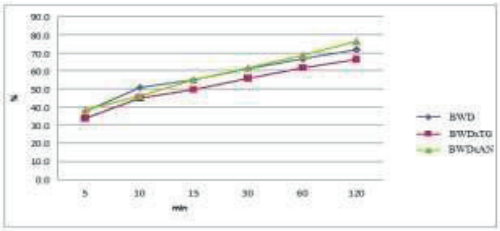

Figure 1. Syneresis of rennet coagulum of goat's milk used for cheese production

At the fifth minute, 37.7% whey was released for BWD, 33.7% for BWD x TG and 38.3% for BWD x AN, after the first hour - 66.7% for BWD, 61.7% for BWD x TG, 68.7% for BWD x AN, and after the 2nd hour - 71.7% for BWD, 66.3%, for BWD x TG and 76.5% for BWD x AN.

The milk of BWD x AN had the best syneresis, and the lowest was that of BWD x TG, which is close to the one obtained by Mihaylova et al. (2000) for the amount of whey released - 71% in local goats, 70% in the milk of BWD breed and 66% in the milk of crossings.

Brine cheeses are the most important type of this product for the Eastern Mediterranean and Balkan countries. These types of cheese can be very similar, but also quite different in terms of technological process, composition, physicochemical and organoleptic properties.

The cheese quality varies depending on the physicochemical composition of the goat's milk and the technology used (Litopoulou-Tzanetaki & Tzanetakis, 1992; Barac et al., 2016; Sulejmani & Hayaloglu, 2018).

The relatively lowest water content was registered in the 24th hour of production from BWD milk (53.34%), while the highest was found in milk of BWD x AN (58.40%) (Table 2).

Table 2. Cheese composition at the 24th hour of production

(n = 6)

Indicators	Groups		
	BWD	BWD x TG	BWD x AN
	x ± Sx	x ± Sx	x ± Sx
Water content, %	53.34±0.730	57.10±2.612	58.40±0.910
Protein, %	15.16±0.186a*	12.54±0.871b**	12.76±0.601b**
Fat, %	24.0±0.829	19.32±0.585	20.64±0.690
Milk fat in dry matter (MFDM)	51.41±1.029	48.86±0.227	49.60±0.792
Salt, %	2.46±0.087	2.44±0.090	2.40±0.080
Titrable acidity, °T	146.67±3.333	139.33±7.024	144.34±4.410
Water in non-fat residue (WNFR), %	70.18±0.317	72.83±1.020	72.97±0.616
Salt in water phase (SWP), %	4.60±0.100	4.30±0.246	4.11±0.177

Note: a - BWD; b - BWD/BWDxTG; *P≤0,05; **P≤0,01

The data for this indicator from the present study are close to those of Barac et al. (2016) for fresh white brined cheese made from goat milk pasteurized at 90°C/10 min (53.29%) and Kondyli et al. (2016) - 54.56%, for goat's milk cheese on the 2nd day of production. Our results are slightly lower than those of Litopoulou-Tzanetaki & Tzanetakis (1992) for white brined cheese made from raw goat's milk (58.0 and 59.4%, respectively, on the 15th and 75th day of production). Sulejmani & Hayaloglu (2018) found a significantly higher water content in

fresh (1st day of production) white brined cheese made from raw (67.4%) and pasteurized at 80°C/2 min goat's milk (67%).

The highest protein and fat content in the cheese at the 24th hour is the highest in the BWD breed - 15.16% and 24.0%, respectively, and the lowest in the BWD x TG - 12.54% and 19.32%, which is higher than that found by Zeng et al. (2007) - 15.9% fat and 11.8% protein for soft cheese (at 24 hours) from mixed milk of Alpine and Nubian goats in the USA.

Soryal et al. (2005) reported values of 12.57% and 12.84% protein in soft milk cheese of Alpine and Nubian goats in the United States and fat, respectively - 15.78% and 15.54% for the same breeds, and Albenzio et al. (2006) found results of 15.4% to 19.4% protein in Cachioricota cheese after one week of maturation from milk of 4 herds of Garganica goats in Italy.

The fat content values in the dry matter in the white brined cheese at the 24th hour from 48.86% at – BWD x TG to 51.41% at BWD are lower than those of Barac et al. (2016) - 57.18% and significantly higher than the data in the studies of Sulejmani & Hayaloglu, (2018) for white brined cheese produced from raw (43.36%) and pasteurized (37.86%) milk.

Cheese ripening process depends on the conditions of the microenvironment in which the lactic acid bacteria develop and the rennet enzyme acts. The microenvironment in the cheese is determined by the content of water, salt and pH. Important for the correct ripening and quality of the cheese are not the absolute, but the relative values of these indicators - water in fat-free residue (WFR) and the salt content in the aqueous phase (SWP) (Upreti & Metzger, 2007).

The water content in the fat-free residue affects the course of biochemical and microbiological processes, and the ability to produce a product with a characteristic taste and aroma (Rearce & Gilles, 1979). Salt content in the aqueous phase and pH at the time of salting also have a great and decisive influence on the normal course of microbiological processes and the durability of the cheese (Lawrence et al., 1987).

The water content in the fat-free residue of cheese at the 24th hour varied in a relatively narrow range - 70.18 ÷ 72.97%, respectively in BWD and BWD x AN. These data are very close to the water content found by Kondyli et al. (2016) in the fat-free residue in goat cheese on the second day of production - 72.88%.

The lowest salt values in goat's white brined cheese were found in BWD x AN - 2.40%, and the highest in BWD - 2.46%. While in the studies of Sulejmani & Hayaloglu (2018) this indicator in fresh cheese is in the range of 2.02 ÷ 2.73%, the data of Barac et al. (2016) for the salt content in goat white brined cheese for the period 10th ÷ 50th day of production are significantly lower - 1.85-2.10%.

The salt content in the aqueous phase in goat cheese at the 24th hour in our study ranged from 4.11 to 4.60%. For goat white brined cheese on the 2nd day of production Kondyli et al. (2016) found 1.47% salt and 2.69% salt in the aqueous phase.

The goat's milk cheese had the highest titratable acidity in BWD breed - 146.67ºT, and the lowest in BWD x TG - 139.33ºT, which is less than the titratable acidity established by Jeleva (2005) - 156.9ºT for white brined cheese of buffalo milk at 24 hours. On the third day of the production of traditional white brined cow's milk cheese with a leaven consisting only of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* or supplement to them of *Lactobacillus paracasei* subsp. *paracasei* (L. casei), Dabevska-Kostoska et al. (2015) found an even lower titratable acidity - 107.8 ÷ 141.1°T (0.97 ÷ 1.27% lactic acid).

The research data of Mallatou et al. (1994) showed that fresh goat Feta cheese had an average water content of 58.5%, 75.6% water in the fat-free residue and a titratable acidity of 144.4°T (1.3% lactic acid).

The cheese composition on the 45th day of production is presented in Table 3.

Compared to the cheese at the 24th hour of production, the water content of the mature white brined cheese from the individual batches decreased slightly and reached values of 52.60%, 54.74% and 55.11%, as the values for BWD breed were by 0.74%, for BWD x TG by 2.36%, and for BWD x AN by 3.29% lower.

Table 3. Cheese composition on the 45th day of production

Indicators	Groups		
	BWD	BWD x TG	BWD x AN
	x ± Sx	**x ± Sx**	**x ± Sx**
Water content, %	52.60±0.865a*	54.74±0.538	55.11±0.595b*
Protein, %	14.60±0.857a*	11.97±0.708	11.95±0.617b*
Fat, %	27.71±1.325	25.05±1.153	25.08±1.183
Milk fat in dry matter, % (MFDM)	58.42±1.924	55.32±2.186	55.84±1.939
Salt, %	2.67±0.064	2.63±0.055	2.77±0.095
Titrable acidity, °T	223.33±14.530a*	243.33±20.078c*	230.0±101b*
Water in non-fat residue, % (WNFR)	72.77±0.643	73.06±0.871	73.58±0.498
Salt in water phase, % (SAP)	5.09±0.185	4.88±0.116	5.03±0.230

Note: a - p<0.05; a-BWD; b - BWD/BWD x AN; c - BWD/TG; *P≤0.05.

Its content was significantly higher in the present study than those found by Peychevski et al. (1986) for mature white brined cheese from the milk of Saanen goats - 49.10 ÷ 50.03%, probably due to the higher acidity of the processed milk and the accelerated syneresis of the cheese mass during the technological process (Gorbatova, 1984).

Unlike the present result, Peychevski et al. (1986) and Kondyli et al. (2016) found an increase in the water content of mature white brined cheese (60th day) from goat's milk by 0.91%, compared to fresh (2nd day of production). The same tendency to increase the water content from 55.83 to 61.84% was found by Miloradovic et al. (2017) for goat white brined cheese, produced by the traditional technology - heat treatment of milk at 65°C/30 min, as in the study of Kondyli et al. (2016). More significant increases in water content in mature goat's white brined cheese - 47.0 to 54.4% and 53.29 to 57.05% were obtained in other studies (Dabevska-Kostoska et al., 2015).

These results from the studies of Dabevska-Kostoska et al. (2015), Kondyli et al. (2016) and Miloradovic et al. (2017) are probably due to the long initial ripening at high temperature - respectively 16-18°C for 15-20 days and 13-15°C for 40 days, which affects the course of biochemical processes in the cheese.

The cited results are in contradiction with our data and those of Balabanova (2015), who found a decrease in the water content in cow's and buffalo's white brined cheese, ripening at 15°C - by 8.3% and 13.4%, respectively.

The highest protein was found in BWD breed - 14.60% and the lowest in BWD x AN - 11.95%, as its values decreased compared to the 24th hour - by 0.56% in BWD, 0.57% in BWD x TG and 0.81% in BWD x AN.

Data from the study by Kondyli et al. (2016) showed a more significant decrease (by 1.44%) in the protein content of mature goat white brined cheese on day 60. Similar results were obtained for white brined Feta cheese (Mallatou et al., 1994) and from goat's milk.

Barać et al. (2016) prove that the protein content in white brined goat's milk cheese decreases during ripening, which coincides with that found in our studies and is due to the fact that ripening takes place in salted brine and the decrease in protein values is due to the diffusion of weakly bound or partially hydrolysed proteins in the cheese.

The highest milk fat values were found in BWD - 27.71%, and the lowest in BWD x TG - 25.05%, which is identical to that indicated by Popović-Vranješ et al. (2016) - 25.3% fat in hard cheese produced in Serbia and less than that obtained by Poveda et al. (2008) - 34.75% and 37% in hard goat cheese from different geographical regions.

There is a relationship between the casein and milk fat content of processed milk and the composition and yield of the cheese. At the same fat content, the increase of casein amount raises the efficiency of using the dry matter in processed milk increases. This reduces the milk fat of the dry matter in the cheese while maintaining the standard requirements. The standardization of milk allows the production of a standard product in terms of milk fat in dry matter and water content (Chomakov & Peychevski, 1974; Peychevski et al., 1986).

The highest milk fat content in the dry matter was registered in the cheese from BWD milk - 58.42%, and the lowest in BWD x TG breed -

55.32%, which is higher than that obtained by Popović-Vranješ et al. (2016) - 42.6% - 51% in mature cheese produced in Serbia.

Peycheski et al. (1986) also obtained a lower content of milk fat in the dry matter (44.20-44.76%) of white brined cheese than the milk of Saanen goats (K/M 0.709-0.738). We attribute this to the much lower ratio in the goat's milk processed by us (0.413 ÷ 0.430 - Table 1).

During ripening, the water in the fat-free residue in the white brined cheese in all three batches studied by us varied in a relatively narrow range and increased slightly from 70.18 to 72.77% (BWD), 72.83 to 73.06% (BWD x TG) and from 72.97 to 73.58 % (BWD x AN). Our results are close to those found by Kondyli et al. (2016) - 72.88 ÷ 74.48%, in contrast to the research of Miloradovic et al. (2017), whose data show a significant increase in WFR in the maturation process - from 67.52% to 74.92%.

The results of the research of Peychevski et al. (1986) show a significantly lower content of WFR in mature goat white brined cheese - 63.35 ÷ 64.03%.

Chen et al. (2010) investigated the technological qualities of Alpine goat milk with different number of somatic cells in the production of semi-soft Colby cheese and also found a low water content in the fat-free residue in the cheese - 63.55 ÷ 64.19%.

The salt amount varies within narrow limits between the individual batches of cheese. Compared to white brined cheese, at the 24th hour of production, the salt content of mature cheese from BWD milk was 0.21%, at BWD x TG by 0.19%, and at BWD x AN by 0.37% more on the 45th day of maturation.

Dabevska-Kostoska et al. (2015) found an almost double increase (from 2.55 to 5.14%) in the salt content of goat white brined cheese on day 40, and Kondyli et al. (2016) - significantly lower values (1.47-3.05% on the 60th day).

The salt content in the aqueous phase in the cheese at the 24th hour and the mature cheese from the three groups in our study varied in a relatively narrow range - from 4.11-4.60 to 4.88-5.09%. The results of Kondyli et al. (2016) are close to the above - from 2.69 to 5.50%, in contrast to the data of Dabevska-Kostoska et al. (2015), which vary in a significantly longer range - from 4.79% to 9.01%. The salt content in mature white brined cheese from the milk of Saanen goats is relatively high in the research of Peychevski et al. (1986) (7.94-7.95%).

The titratable acidity during ripening increased in the milk cheese of all three groups of animals and is close to that found by Gerchev et al. (2004) - 221.5ºT for white brined cheese from milk of goats of different breed and age composition, raised in the region of the Central Balkan Mountain. These and our results are lower than the established titratable acidity in mature Saanen goat cheese in the studies of Peychevski et al., (1986) - 274.4 ÷ 284.4°T. In contrast to the above data, Dabevska-Kostoska et al. (2015) found significantly lower titratable acidity in goat white brined cheese on the 40th day - 147.8°T. The titratable acidity of Feta cheese from goat's milk on the 60th day of production is even lower - 111.1°T (Mallatou et al., 1994).

CONCLUSIONS

1. Goat's milk processed into white brined cheese from the studied goat breeds, such as Bulgarian White Dairy and its crossings with Toggenburg and Anglo-Nubian raised in one herd under the same production conditions is characterized by the lowest content of dry matter (13.03%), milk fat (4.91%), dry fat-free residue (8.32%), protein (2.97%), casein (2.03%), calcium (0.139%) and density (28.2 ºG), in Bulgarian White Dairy breed compared to its crossings.

2. The hygiene indicators were within acceptable limits and comply with Regulation (EC) No 853/2004.

3. The slowest curdling (294 s) was observed in milk from BWD, and the fastest in milk from BWD x AN (283 s), with the best syneresis observed.

4. The lowest water content at the 24th hour of production was observed in the milk cheese of Bulgarian White Dairy breed (53.34%), as the highest protein and milk fat values (15.16%, 24.0%) were observed in the batch produced from the milk of the same breed.

.In the mature white brined cheese on the 45th day of production, the water and protein content decreased in all three batches compared

to the 24-hour cheese, while the values for milk fat, fat content in the dry matter, water in the fat-free residue and salt in the aqueous phase increased.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

DEVELOPMENT OF NUTRITIONALLY ENHANCED PASTA WITH DIFFERENT ORGANIC POWDERS

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Abstract

Nutritionally enhanced pasta was developed by adding powders freeze dried by tomato (Lycopersicon esculentum L. var. Coeur de Boeuf, wild garlic (Allium ursinum L.) and basil (Ocimum basilicum L.). This study wants to development, testing and evaluation of novel natural food additives, and their application in different products, including quality and sensorial assessment of their preserving functions. The aim of this study was to investigate the addition of organic powders to the pasta formulation, improving with powders freeze-dried by Coeur de Bœuf tomato, wild garlic and basil. In addition, the effect of powders on the sensorial properties, and cooking quality of the pasta samples was also determined in this study. The organic powders were added to the pasta formulations of 1% of the total weight of the wheat flour. The results showed that the addition of different organic powders to the pasta formulation resulted in a significant enriched in the colour, swelling volume, and weight gain.

Key words*: basil, pasta, sensory analyses, tomato, wild garlic.*

INTRODUCTION

Pasta becoming popular in current lifestyle, because are healthy, tasty and convenient for transportation and preparation. The assortment range of pasta has had a beautiful evolution in recent years. On the market, the product offer has diversified by launching new brands and types of products, manufacturers have focused their attention on all consumer segments and added value to the raw material and consumers' perception of pasta has improved through a new approach (Dragomir et al., 2017).

With the diversification of supply, the lifestyle and increase of purchasing power, the consumption behaviour of pasta has changed (Bahaciu et al., 2019; Dragomir et al., 2020). The Romanian consumer tends to modernize, preferring quality products, easy to prepare, tasty and healthy. We can say that a very important role is played by the younger generations, who prefer light and innovative menus. Romanians abroad, who on their regular visits to the country, bring this habit of consumption from the country of adoption.

Pasta like dish, present three main advantages: it is prepared quickly; are very economical and healthy. These three main qualities make pasta a product approved by more and more consumers and increase its potential in the future.

From an environmental point of view, pasta is a simple food that has a low environmental impact, with a carbon footprint that is much less than animal products. Life cycle analyses of pasta products, from production to retail, have shown that the most significant impact on the environment occurs during the phase of wheat cultivation - 80% of the ecological footprint, approximately 60% of the carbon footprint, and the entirety of the water footprint (Webb et al., 2019; Sustainable Farming, 2017).

Tomato (*Lycopersicon esculentum* L. var. *Coeur de Bœuf*) is an important vegetable crop grown in many countries across the world for fresh market and multiple processed forms (Mutari et al., 2011; Hassen et al., 2019). Tomatoes are widely consumed in the world and as a fruit of limited durability, short agricultural season. Much of the tomato produced for industrial purposes is transformed into concentrated pulp, which is reconstituted throughout the year, mainly during the off-season (Munhoz Silveira et al., 2019; Ochida et al., 2019; Demissew et al., 2017). But, fresh tomato has a limited storage life 2-3 weeks under ambient temperature and cannot be

stored over extended periods (Dobrin et al., 2019). To minimize after harvest losses, the tomato is processed in the forms of paste, juice, ketchup, sauce, and purée. One of the industry's biggest challenges is to produce in a sustainable and effective chain to simultaneously fulfill the cost reduction and quality improvement demands (Koufiotis et al., 2016; Munhoz Silveira et al., 2019). Possible preservation methods of tomato include physical (application of heat, freeze-dried, irradiation, and soundwave) and chemical preservatives or combinations of those different means of methods (Fellows et al., 2000). Among these preservation methods, thermal processing is one of the most common and effective means.

During the processing and subsequent storage of products the content of carotenoids falls down and the colour changes (Kumar et al., 2015). Recent studies have indicated the potential health benefits of a diet that is rich in tomatoes. Commonly consumed in daily diets, are a major source of antioxidants, which have a greater contribution to a well-balanced healthy diet with the right proportion of vital nutrients such as minerals, vitamins, essential amino acids, sugars, lycopene, and other carotenoids and dietary fibers (Jaramillo et al., 2007; Sgherri et al., 2008). Lycopene, a major carotenoid without pro-vitamin an activity, present in red tomatoes, is considered responsible for their beneficial effects (Reboul et al., 2017; Kumar et al., 2015; Shi et al., 1999; Rao et al., 1998). Different studies have suggested a protective role for lycopene, an antioxidant carotenoid, in the prevention of stress including environmental stress. Tomatoes and tomato products are the major dietary source of lycopene (Kohlmeier et al., 1997; Charu et al., 1999).

Allium ursinum - known as ramsons, buckrams, wild garlic, broad-leaved garlic, bear leek, or bear's garlic - is a wild relative of chives native to Europe and Asia. Wild garlic is best picked in spring, before the flowers are too developed. Usually find it in shaded woodland near water. It`s recognized by its specific, wild taste, a garlicy and very aromatic taste.

In European traditional medicine *Allium ursinum,* has been generally recommended as digestive stimulant, antimicrobial agent, removing toxins from the body, and to prevent cardiovascular diseases (Treben, 1992; Macků & Krejča 1989; Leporatti and Ivancheva, 2003). It was often applied as a remedy in respiratory problems, such as common cold with fever or bronchitis.

In recent years there has been a growing interest in its use as a dietary supplement and food. It has become a practice for wild garlic leaves to use in cuisine. Fresh leaves can be eaten raw or cooked, and as a kind of pesto. They are often added to soups, gnocchi, risotto, ravioli, and as a spice to flavour hard cheeses or spreads based on cottage cheeses. Leaves and flowers can be used as a garnish to salads, while wild garlic bulbs can be used like common garlic (Sobolewska et al., 2015).

Researchers in the nutritional field and the food industry have rediscovered this plant, and are researching it due to its powerful antioxidant properties. These properties are due to many substances, including some vitamins, flavonoids, terpenoids, carotenoids, phytoestrogens, minerals and volatile compounds. Wild garlic contains polyphenolic compounds (Gîtin et al., 2012), substances with antibacterial activity, use like an alternative preservative ingredient to protected against pathogenic bacteria in food (Jensen et al., 2014).

Basil (*Ocimum basilicum* L.) belongs to aromatic plants due to their volatile compounds presented especially in leaves and flowering tops. These basil parts are used since antiquity for food preservation, flavouring, and as medicine, because of high antioxidant, antibacterial and antifungal activity of volatile oils, being good sources of natural antimicrobial and antioxidant agents, with possible application in food industry, cosmetics or medicine (Avetisyan et al., 2017).

By freeze-dried basil retains the characteristics intense colour and flavour. Freeze-dried basil powder it is aromatic, slightly sweet, with spicy notes in taste. Because, it has a great capacity to rehydrate in the presence of water from the dough, the original character, such as the taste, colour and aroma specific to the basil, is present in the new preparation. Added the powder from the lyophilized basil aromatizes to the dough balances the taste and increases the preservation of final product. (Dragomir et al., 2020)

MATERIALS AND METHODS

Develop pasta products

The purpose of this work is to incorporate in pasta, powders from indigenous plants, with high aromatic, coloristic profile and nutritional value. The goal was to find a vector food that would bring to the consumer's diet the benefits of tomato *Coeur de Bœuf*, wild garlic (*Allium ursinum* L.) and basil (*Ocimum basilicum* L.) organic powders on studied.

Within the study, 4 assortments of organic pasta were obtained, presented in Table 1.

Table 1. Description of the pasta samples elaborated in the study

Sample	Main ingredients
P-WG	Pasta enriched with 1% wild garlic powders
P-B	Pasta enriched with 1% basil powders
P-T1	Pasta enriched with 1% *Coeur de Bœuf* tomato powders
P-T3	Pasta enriched with 3% *Coeur de Bœuf* tomato powders

The flour intended for obtaining pasta comes from organic common wheat varieties and tomato juice is bought from specialty stores. The ecological powders used in recipes were obtained at the Research Center for Studies of Food Quality and Agricultural Products within the University of Agronomic Sciences and Veterinary Medicine Bucharest, within the *SusOrgPlus: Intelligent food processing chains, natural additives and colourants*, which aims to develop advanced processing technologies for organic products and their by-products, in order to reduce raw material losses and increase their economic value.

Determining consumer acceptance.

Pasta samples were cooked in water to optimum cooking time, and after draining for 2 min, they were served to the panellists. The sensory test panel consisted of ten panellists who were trained academic staff. The sensory properties (appearance, texture, colour, flavour & smell, taste) of fresh prepared were evaluated by forty panellists. For the sensory evaluation, five-point hedonic scale was used, where 5: like very much, 4: like moderately, 3: neither like nor dislike, 2: dislike moderately and 1: dislike very much for each attribute. To achieve the sensory profile, the evaluator completed a form for each test. After scaling the average values of the 5 attributes and their representation on a spider diagram (Lawless et al., 2010).

Stability of organic fresh pasta

Sensory analysis of innovative organic food products obtained during the study - was performed in the Laboratory of quality control of agri-food products, the Faculty of Engineering and Management of Animal Productions at the University of Agronomic Sciences and Veterinary Medicine of Bucharest.

The influence of organic ingredients on the sensory quality of the tested products was evaluated by the intensity of flavour and smell, taste and aftertaste attributes. Averages were made of the values recorded on the attributes of each evaluator, for each product.

The technological-culinary examination is performed by determining the boiling behaviours. The analysis of the boiling behaviours of pasta is a final criterion for their acceptance as food in current human diet. Regarding the boiling behaviours of pasta, some differences persist among researchers, industrial operators and consumers regarding the main factors for evaluating quality indicators (Mohan, 2002). The determination of the behaviour of boiling pasta is performed according to STAS 756/1/A2-1999, STAS 756/1/A1-1997.

During the evaluation of the stability over time of pasta chilled enriched with organic powders, the following were considered: increase the volume of pasta when boiling; the consistency of the pasta, respectively the tendency of agglomeration; the colour of pasta, during and after boiling.

Determining the nutritional and energy value of organic products

To determine the nutritional value of innovative organic food products, their energy value was calculated, depending on the composition in nutrients (proteins, lipids, carbohydrates, fiber), for each ingredient, for 100 g of product and the amount of finished product. The nutritional and energy value was calculated using a nutritional calculation tool, the Softfedima Program (http://softfedima.ro/).

RESULTS AND DISCUSSIONS

This study to development, testing and evaluation of novel natural food additives and their application in different products, including quality and sensorial assessment of their preserving functions. A new assortments of pasta enriched with freeze dried by wild garlic (*Allium ursinum* L.) and basil (*Ocimum basilicum* L.) and Coeur de Boeuf powder was made and investigate, the effect of powders on the sensorial properties, and cooking quality of the pasta samples was also determined in this study. A powder was obtained within the SusOrgPlus project at the Research Center for the study of the quality of USAMV agri-food products in Bucharest.

Develop pasta products

Pasta is a staple food in many countries all over the world. Many research studies have been conducted around the world to develop pasta products with nonconventional ingredients and added functional properties to meet the demand of health conscious consumers (Nilusha et al., 2019).

Pasta is a product obtained from fresh pasta dough, unfermented, shaped into various shapes and refrigerated. The dough is prepared from wheat flour and water, with or without additives (Adegunwa et al., 2012).

The technological process of obtaining the product includes the following steps (Figure 1):

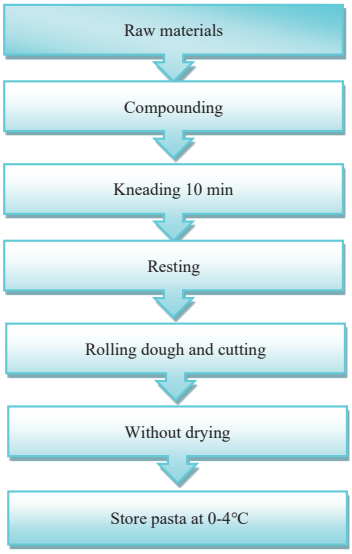


Figure 1. Conventional pasta production process

The innovative ingredients used in our study included:

- Organic wild garlic powder: it`s a fine, green powder with a specific, characteristic, aromatic smell of onion and garlic.
- Freeze-dried organic basil powder: it`s a fine powder, of intense green colour, with intense aroma, with specific notes of smell and taste.

➢ Organic *Coeur de Bœuf* tomato powder: it`s a fine powder, of intense red colour, with flavour weak, with specific notes of smell and taste.

Within the study, a new assortment of organic pasta was obtained, presented in Table 2.

Table 2. Main ingredients

Sample/ ingredients (%)	Organic powders	Wheat flour, %	Water, %	Tomato juice, %
P-WG	1% wild garlic powders	66	33	-
P-B	1% basil powders	66	33	-
P-T1	1% *Coeur de Bœuf* tomato powders	61	12	24
P-T3	3% *Coeur de Bœuf* tomato powders	61	12	24

For the preparation of pasta enriched with tomato *Coeur de Bœuf* powder, part of the water was replaced with organic tomato juice and the percentage of added powder was increased by 1% and 3%.

Preparation of fresh pasta: organic wheat flour was properly prepared and the organic powders were dosed. Organic powders were previously hydrated in small amount of water, to be incorporated more easily in dough. The pasta dough was kneaded for 10 minutes until easy-to-shape dough is obtained. The dough is rest for a few minutes, and then is spread until a thickness of 2-3 mm is obtained; it is cut in the form of fidelities with a thickness of average 3 mm, and twist to form nests. The pasta thus shaped is spread in a layer and subjected to drying at room temperature for 2 hours. The pasta is packed properly and kept at a temperature of 4ºC.

Organic *Coeur de Bœuf* tomato powder is very fine, incorporates very well, but requires a larger amount to be added if the goal is to obtain a more intense colour.

Determining consumer acceptance

The sensorial evaluation of product was carried out in order to observe the impact of organic powder incorporation in dough pasta, on its

sensory characteristics. The panellists evaluated the products for appearance, texture, colour, flavour and smell, taste and overall acceptability using a 5-point hedonic scale ranging from 5 (like extremely) to 1 (dislike extremely) for each sensory characteristic. After scaling the average values of the 5 attributes and their representation on a spider diagram, the following representation was obtained.

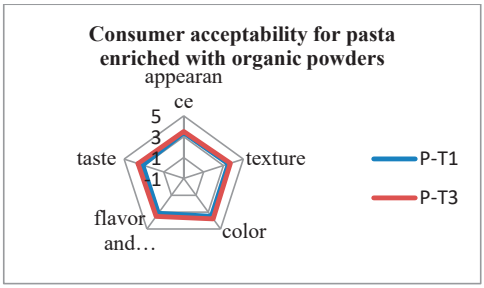


Figure 2. Consumer acceptability scores on a 5-point Hedonic scale for pasta enriched with tomate organic powders

Following the analysis of the P-T1 and P-T3 sample after boiling, the following aspects, were observed: the pasta has a slightly reddish, uniform color, the product shows no traces of flour, when boiling the product has increased its volume and the water has not colored during boiling, the boiling water is clear and free of starchy sediment.

At pasta enriched with tomato *Coeur de Bœuf* powders (P-T1) sample, the most appreciated attributes were texture, color and appearance. For P-T2 sample, the most appreciated attributes were color, texture and taste.

The taste of tomato, brought into the product by the addition of freeze-dried tomato powder was intense and felt on both the freshly boiled product and the cold product.

Sensory assessment results of pasta are given in Figure 2. The panelists gave the best scores to both products, sample P-T3 enriched with 3% tomato powder it was prefered. It is recommended to increase the level of tomato powder added in the pasta, so as to improve the color and taste.

The overall acceptanbility results indicated (Figure 4) that all pasta samples had a good sensorial score, but the most preferable one was the pasta enriched with 3% tomato *Coeur de Bœuf* powder.

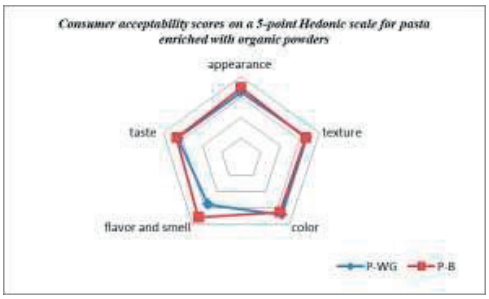


Figure 3. Consumer acceptability scores for pasta enriched with organic wild garlic powders (P-WG) and basil powders (PB)

Following the analysis of the P-WD sample after boiling, the following aspects, were observed: the pasta has a slightly greenish, uniform color, the product shows no traces of flour, when boiling the product has increased its volume and the water has not colored during boiling, the boiling water is clear and free of starchy sediment. The most appreciated features were the texture and color, each marked with 3.37. The taste of wild garlic, brought into the product by the addition of freeze-dried wild garlic powder was more intense on the freshly boiled product and became less pronounced on the cold product. This behavior was also dictated by the type of flour used in the preparation. Overall acceptability obtained an average value of 3.20.

For pasta enriched with basil powders (P-B), the most appreciated characteristics were flavor and smell (3.54) and apearance (3.46). The taste of basil, brought into the product by the addition of basil powder was intense and felt on both the freshly boiled product and the cold product. Taste it was influenced by the what type of flour it was used. Overall acceptability obtained an average value of 3.37.

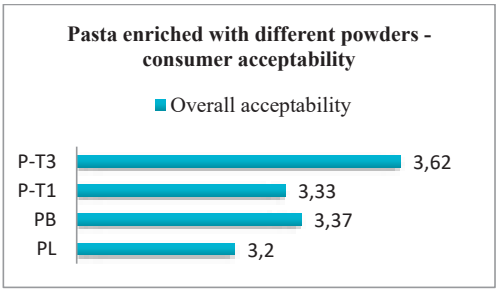


Figure 4. Overall accepability obtained for pasta enriched with organic powders

Samples were selected on the basis of sensory and quality, it was subjected for assessing the storage stability. Prepared products were packed in high density polyethylene packs and stored at refrigeration temperature for shelf life study.

Color is one of the most important quality properties for the acceptability of food due to its relation with product freshness and flavor expectations and therefore has a direct effect on consumers' perceptions. Because of the positive impact on the consumers, production on colored pasta have gained attention in the recent years..

Because the reticent to the use of synthetic colorants in foods has increased, the use of natural colorants has the advantages of being readily accepted by the consumers, considered to be safe and not chemical.

Stability of organic fresh pasta

The optimal cooking time for pasta depends on the preferences and how the consumer is accustomed to consuming them. The main characteristic that must be maintained after boiling, for Western Europe, especially in Italy, is a certain consistency, called "*al dente*", which according to most consumers in Eastern Europe corresponds to insufficient boiling. In the tradition of the Romanian people, pasta is eaten with pleasure when it is softer. For this reason, each producer writes on the pasta packaging a cooking time recommended, respected or not by consumers, but which remains an indication for preparation (Iancu et al., 2014).

During the evaluation of the stability over time of fresh pasta enriched with organic powders, it was in considered the following: increase the volume of pasta when boiling; the consistency of the pasta, respectively the tendency of agglomeration; the colour of pasta during and after boiling.

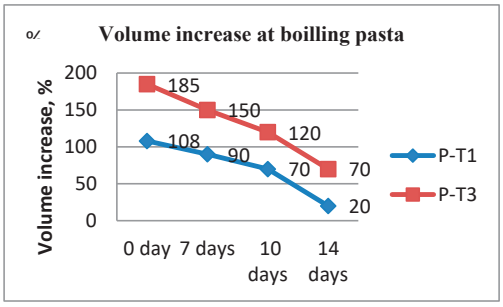
Cooking quality: Weighed samples (10 g) of chilled pasta were cooked in 250 ml boiling water. Then, rinse with cold water. Optimum cooking time, firmness of pasta and solids lost to cooking water was assessed using standard method. Per cent water absorption and volume expansion ratio was calculated from increase in weight and volume, on cooking of pasta for optimum cooking time.

The optimum cooking time was established by boiling the pasta in distilled water until the white center core of the pasta strand disappears, indicating that the starch at the center has gelatinized. For the two samples taken in the analysis, the optimal boiling time was 10-14 minutes.

Overall acceptability of stored pasta was evaluated on the basis of sensory attributes (appearance, colour, texture, stickiness, flavour and smell, taste) by a panel of trained judges. Following the determination of the behaviour of the pasta during the analysis period, they behaved very well, they were stable.

Fresh pasta unprepared showed stability during storage. They developed a tendency to moisten after 10 days of storage in refrigerated conditions becoming very sticky and forming agglomerations after the 10th day of analysis. On day 14, the pasta showed a strong tendency to agglomerate and began microbiological degradation.

The storage period had no significant effect on the minimum cooking time of the enriched pasta stored as the storage period progressed; the time required for cooking the pasta increased, however, the increase was very small.



Fagure 5. Volume increare at boilling pasta enriched with 5organic tomate powders

It is observe that the P-T3 sample shows a higher increase in volume compare to the P-T1 sample. According to color measurements and evaluations of the panelists, adding tomato powders in pasta provided an appealing reddish tone.

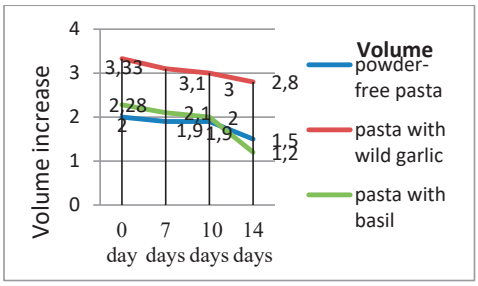


Figure 6. Volume increase at boilling pasta enriched with organic tomate powders

It is observed that the PL sample had a very good behavior during the preparation and corresponded to our expectations. The PB test had a good evolution, until day 10, after which there was a change in the characteristics of both unprepared pasta.

After cooking, it was observed color loss in boiling water, the pasta acquiring a brighter colors, a specific light yellow. Similar results were found by in specialty literature (Fradique et al., 2010; Zourai et al., 2011; Özyurt G. et al., 2015).

Shelf life is low, in the case of refrigerated pasta: simple storage for 14 days, and through packaging in modified atmosphere, extend storage period up to 2-3 months.

Nutrient Content. For the calculation of the nutritional value, technological losses were taken into account, so that the energy value kcal per 100g of pasta enriched with organic powders so that it is correctly calculated (Table 3).

Table 3. Nutritional declaration for pasta enriched with organic plant powders

Nutritional value for 100 g product					
	P-T1	**P-T3**	**PM**	**PB**	**PL**
Energy	906.7 kj	921.9 kj	931.1 kj	972.5 kj	872.5 kj
	214.7 kcal	218.3 kcal	220.5 kcal	230.7 kcal	206.6 kcal
Total fat	1.3 g	1.3 g	1.3	1.7	1.2
Saturated fat	0.6 g	0.6 g	0.7	0.6	0.6
Carbohydrates	39.8g	39.9 g	40.4	41.1	38
Sugar	1.9 g	2.7 g	0.7	0.6	0,6
Fiber	8.3 g	8.5 g	8.8	11.5	8.1
Protein	7.4 g	7.5 g	7.4	7	6.9
Salt	0.1 g	0.1 g	0.1	0.1	0.1
Allergens: the product contains GLUTEN					

The addition of powder in a higher percentage causes a change in the content of dietary fiber (fiber: P-T1-8.3g, P-T3 -8.5g) and an increase in the percentage of protein (protein: P-T1 - 7.4 g, P -T3 - 7.5 g). These values show the influence of the addition of *Coeur de Bœuf* tomato powder, respectively high values of protein and dietary fiber. In terms of energy value, the values have not changed much, but there is a significant increase in energy value as the percentage of *Coeur de Bœuf* tomato powder increases.

The addition of basil powder causes an increase in the percentage of dietary fiber in pasta (PB - 11.5 g), compared to addition on wild garlic powders in pasta (PL - 8.1 g).

These values show the influence of the addition of organic plant powder, determ a high values of protein and dietary fiber in freshly made pasta. The energy values for pasta enrich with basil powders increase very much, with the addition of powder .

CONCLUSIONS

Cooking quality of enriched pasta sample was good for the technological attributes. Pasta samples enriched with tomato powders had also desirable sensory properties as indicated by the panellists. Consequently, on the basis of these results, pasta enriched with organic powders may have a great potential for the industry to develop functional products.

Pasta with the addition of wild garlic powder and with the addition of lyophilized basil powder were highlighted by a specific colour, a light green in the PL sample and intense green in the PB. Each type of powder imprinted the original character of the product, such as specific taste, colour and aroma, attributes appreciated by evaluators. Pasta with organic freeze-dried wild garlic powder is recommended to be eaten plain or with different sauces. Pasta with freeze-dried basil powder is recommended to be eaten plain or with sauces (pesto sauce).

For a intense colour, it is recommended to add a percentage higher than 1% freeze-dried organic plant powders in dough. In the case of adding wild garlic powder in pasta dough, it must be taken into account that a higher percentage can influence the taste and aroma of the finished product. Basil powder is extremely versatile and can be added in percentages greater than 1%, but the taste and aroma will be

significantly highlighted. One thing worth noting is that when processing basil powder must take into account that due to the high fiber content it needs an additional amount of water in the preparation.

ACKNOWLEDGEMENTS

The authors acknowledge the financial support for this project provided by transnational funding bodies, being partners of the H2020 ERA-net project, CORE Organic Cofund, and the cofund from the European Commission. This work was supported by a grant of the Romanian Authority for Scientific Research and Innovation, CCCDI - UEFISCDI, project number 4/2018 ERANET-COREORGANIC SusOrgPlus, within PNCDI III.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

BEE POLLEN METHANOLIC EXTRACTS: TOTAL POLYPHENOLS CONTENT AND ANTIBACTERIAL ACTIVITY

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Abstract

The aim of this work was to study the total phenolics content and antibacterial activity of bee pollen samples from different sources. Total polyphenols content was quantified according to the Folin-Ciocâlteu spectrophotometric method using GA as standard and the results were expressed in terms of mg GAE/g pollen. Furthermore, the antibacterial activity of bee pollen samples from different sources were collected and investigated against multiple bacterial strains, as follows: five gram-positive Staphylococcus aureus ATCC 6538P, Bacillus cereus ATCC14579, Bacillus laterosporus 6932, Paenibacillus larvae 9820 and Paenibacillus alvei 13253, and four gram-negative Escherichia coli ATCC 10536, Pseudomonas aeruginosa ATCC 27853, Salmonella enteritidis ATCC 13076, Salmonella typhi ATCC 14028 and the yeast Candida albicans ATCC 90028. The samples with predominant pollen in Brassicaceae and Rosaceae possessed high levels of polyphenols content, whereas the samples predominant in Fabaceae showed lower levels of polyphenols. Regarding the antibacterial activity, our results revealed that most of the strains were inhibited by the ½ and ¼ dilutions. The gram-negative bacteria and the yeast Candida albicans proved to be resistant to all bee pollen methanolic extracts. Our results showed that bee pollen samples have strong antibacterial activity against gram-positive bacteria.

Key words*: antibacterial activity, bee pollen, methanolic extracts, palynology, total polyphenols.*

INTRODUCTION

Nowadays, there is an increasing interest in the consumption of honey bee products (bee-collected pollen, beebread, honey, royal jelly, propolis etc.) along with their implementation as functional foods and alternative medicines (Gardana et al., 2018; Margaoan et al., 2019; Daoud et al., 2019; Rzepecka-Stojko et al., 2018).

Bee collected pollen (BCP) contains all the necessary nutrients and phytochemicals which makes it an important source of protein, lipids, polyphenols, macro and microelements, as well as amino and fatty acids (Margaoan et al., 2014; Rzepecka-Stojko et al., 2015). Multiple studies have demonstrated that an enriched diet in polyphenols may fight against multiple diseases, such as cancer (Markiewicz-Żukowska et al., 2013; Sobral et al., 2017; Maric et al., 2020; Al-Yousef et al., 2020), diabetes (Laaroussi et al., 2020; Mohamed et al., 2018), cardiovascular diseases (Rzepecka-Stojko et al., 2015), atherosclerosis (Rzepecka-Stojko et al., 2017) and recently for environmental cleanup (Maric et al., 2020). Furthermore, the chemical composition is strongly correlated to botanical and geographic origin, climate conditions during collection, as well as bee's plant preferences in terms of pollen gathering (Campos et al., 2010).

In a recent study, Kostic and his collaborators determined the phenolic profile and antioxidant properties of methanolic and ethanolic extracts of monofloral bee-collected sunflower pollen from Serbia. Their results showed that the methanolic extract had a higher phenolic content compared to the ethanolic extract. Comparatively, the concentration of quantified phenolic compounds proved to be higher in the ethanolic extract (244.44 mg/kg DW) than in

methanolic (200.58 mg/kg DW). According to their results the methanolic extract showed a higher scavenging activity against ABTS (95.5% inhibition) compared to ethanolic (75% inhibition). The same was noticed for ferric reducing ability. This may be due to the different composition (i.e. proteins) in the ethanolic extract which may reduce the antioxidant capacity (Campos et al., 2003).

Bakour et al. (2019) evaluated the potential antioxidant activity, total phenolics, flavones, and flavonol content of hydro-ethanolic extracts of pollen from fourteen plants. Their result showed that the total phenolic contents varied from 9.20 ± 0.12 mg GAE/g in the *Malva sylvestris* pollen samples, to 71.20 ± 0.72 mg GAE/g in the *Mentha spicata* pollen samples.

The antibacterial activity of monolforal and polyfloral BCP has been extensively evaluated in numerous studies. Recently, Velasquez et al. (2017) demonstrated the positive correlation between the chemical properties and botanical origin of several BCP samples from Chile. Their study showed that the samples predominant in *Brassica* sp. and *Galega officinali*s an abundant source of antioxidants and antibacterial compounds. The same was noticed in the case of *Corylus avellana* BCP collected from different locations in Slovakia (Nikolaieva et al., 2019).

MATERIALS AND METHODS

Palynological analysis

For the palynologycal analysis the methods described by Louveaux et al. (1978) and Almeida-Muradian et al. (2005) were adapted for the bee collected pollen samples. A sample of 2 g, corresponding to approximately 200 pollen pellets, was considered to be represent-tative for botanical origin determination. From each sample, one microscopic slide was prepared without acetolysis, by dissolving and washing the pollen in diluted H_2SO_4 (0.5%) and colored using a mixture of glycerine - gelatin - fuxine for permanent preparation (Louveaux et al., 1978). Slide examination was performed using a Nikon Eclipse 50i optic microscope at 1000× magnification (for identification) and 400× magnification for counting. Five hundred pollen grains were counted from every slide, and percentages of different botanical species were calculated.

Total phenolic content

The total polyphenol content of the bee polyfloral pollen samples taken in the study was determined by the Folin-Ciocâlteu spectrophotometric method using gallic acid as a reference standard (Singlenton et al., 1999; Meda et al., 2005).

The method was adapted for the Biotek SynergyHT multidetection spectrophotometer, with 96-well plate, the volumes used respecting the stoichiometry of the original method. A volume of 25 µl of methanolic pollen extract was mixed for 5min with 125 µl of 0.2N Folin-Ciocâlteau reagent, then 100 µl of Na_2CO_3 solution (75 g/L) was added. The obtained mixture was incubated for 2 hours at room temperature in the dark. The absorbance was read at 760 nm compared to a control (80% methanol).

Antibacterial activity

The antibacterial activity of methanolic bee pollen extract was tested on 10 bacterial strains grouped as follows: 5 gram-positive bacterial strains: *Staphylococcus aureus* ATCC 6538P, *Bacillus cereus* ATCC 14579, *Bacillus laterosporus* 6932, *Paenibacillus larvae* 9820 and *Paenibacillus alvei* 13253, 4 strains of gram-negative bacteria *Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enteritidis* ATCC 13076, *Salmonella typhi* ATCC 14028 and a yeast *Candida albicans* ATCC 90028. The strains used in this study came from the Universities' strain collection of the Microbiology laboratory.

Serial dilution method (MIC- minimum inhibitory concentration)

The minimum inhibitory concentration (MIC) of methanolic bee pollen extracts was achieved in 96-well ELISA-type microplates.

100µl of sterile Muller-Hinton nutrient broth was introduced into each well. From bee pollen extracts of 15% concentration successive dilutions were made in the range 1/2-1/32. 100 ml of these dilutions were introduced into each well over the Muller-Hinton medium. This was followed by the addition of 10 µl of bacterial suspension with a density of 0.5 on the McFarland scale. The antibiotic Streptomycin was used as positive control (100 µl) and as

negative control the solvent used to extract the active ingredients from pollen (MeOH 70%). Afterwards, the plates were thermostated with the lid closed at 37ºC for 24 hours.

Statistical analysis

Three different replicates of each BCP samples were assayed. Statistical differences between samples were estimated using ANOVA (one-way analysis of variance; Tukey's multiple-comparison test; GraphPad Prism version 4.0, Graph Pad Software Inc., San Diego, CA, USA). A probability value of $p<0.05$ was considered to be statistically significant. We also used principal components analysis (PCA) to verify the relationships between the analysed variables and BCP samples using XLSTAT software (Addinsoft, New York, NY) and PAST software package (version 2.17, Oslo).

RESULTS AND DISCUSSIONS

Palynological analysis

The palynological analysis of the bee pollen samples used in this study was previously determined (Margaoan et al., 2014).

According to the palynological analysis, all pollen samples proved to be polyfloral, with six plant families found to be predominant. In summary, the Rosaceae family was predominant in P1-4, P6 and P11, Fabaceae in P5, P10 and P16, wehreas Asteraceae was predominant in P7 and P8. Sample P13 had Brassicaceae (*Brassica* sp.) as dominant plant family, whereas P14 had Ericaceae (*Calluna vulgaris*) and P15 Salicaceae (*Salix* sp.).

Total phenolic content

The total polyphenols in the studied samples have an average concentration of 5.92 ± 0.12 mg GAE/g sample and vary between a minimum of 2.46 ± 0.04 mg GAE/g (sample P11) and a maximum of 8.87 ± 0.03 mg GAE/g (sample P6) (Figure 1). It should be noted that both samples (P6 and P11) have pollen predominantly from the Rosaceae Family. The major difference is the dominant pollen species: P6 - *Prunus* sp. (83%) and in P11 - *Rosa canina* (48%). Pollen samples P1, P4, P6 and P13 showed a higher content of total polyphenols (>7 mg GAE/g) explained by the presence of *Brassica* sp. pollen. The dominant pollen of Fabaceae family from samples P5, P10 and P16 determined a low content of total polyphenols reaching a maximum of 5.66 mg GAE/g.

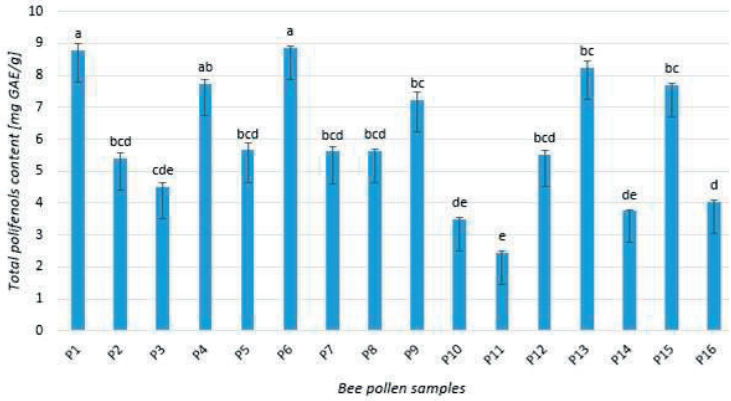

Figure 1. Total polyphenol content of bee pollen samples. Results are given as the mean ± SD (n = 3). Values with different letters (a - e) are significantly different ($p < 0.05$), using ANOVA Tukey's multiple-comparison test. P1−P16, samples of multifloral pollen

Similar results were reported by Stanciu et al. (2012), which showed that the higher TPC was noticed in samples with dominant pollen of *Calluna vulgaris* (4.36 mg GAE/g), *Salix* sp. (8.12 mg GAE/g), *Fillipendula ulmaria* (7.44 mg GAE/g), *Brassica* sp. (8.44 mg GAE/g). Kroyer and Hegeduș (2001) reported the TPC in the range of 7.4-9.7 mg GAE/g sample, whereas Mărghitaș et al. (2009) determined values between 4.4-16.4mg GAE/g sample. Bee pollen samples from SE Brazil studied by Negri et al. (2011) showed a TPC between 1.6

± 0.03 and 2.3 ± 0.03% GAE/g dry pollen, whereas Morais et al. (2011) reported that Portuguese bee pollen samples had a TPC between 10.5-16.8 mg GAE/g extract. Very high values for the TPC published by Freire et al. (2012) with limits between 41.5 ± 0.2 and 213.2 ± 1.1 mg GAE/g, by using etanolic pollen extracts. High values for these compounds were also reported by Menezes et al. (2010), 14.31 ± 0.1 - 132.38 ± 0.7 mg GAE/g sample. Bonvehi et al. (2001) using as extraction solvent of 70% methanol obtained an average of 1.24 ± 0.2 mg GAE/g sample value lower than the TPC determined in the present study. Comparatively, Pascoal et al. (2014), determined high values for this parameter 18.55-32.15 mg GAE/g pollen, using commercial pollen from Portugal and Spain.

The 16 pollen samples analyzed in terms of TPC had values between 2.46 mg GAE/g sample and 8.87 mg GAE/g sample (Figure 1). Comparing the results, it can be seen that the pollen from the Rosaceae family had the highest values P6 (8.87 mg GAE/g sample), P1 (8.80 mg GAE/g sample), P4 (7.74 mg GAE/g sample). These were followed by samples predominant in Brasicaceae and Salicaceae, respectively.

Antibacterial activity

The results of the antibacterial activity of alcoholic bee pollen extract on the Gram-positive bacterium *S. aureus* ATCC 6538P are presented in Figure 2. The diameter of inhibition was between 5.33 mm and 12.33 mm. The negative control used showed no antibacterial activity, and the positive control Streptomycin had an inhibitory diameter of 18 mm. Comparatively, *B. laterosporus* showed a higher susceptibility to the treatment with pollen extracts, the inhibition diamater zone having values between 6.67 and 12.00 mm and the bacterium not being so sensitive to the positive control (18 mm inhibition diameter). The gram-positive bacterium *P. alvei* was the most sensitive to the action of pollen extracts, the diameter of the inhibition zones having values between 8.67 and 21.33 mm. Furthermore, this bacterium proved to be resistant to the positive control (Streptomycin) The Gram-positive bacteria *P. larvae* 9820, as well as the 4 strains of Gram-negative bacteria: *E. coli* ATCC 10536, *P. aeruginosa* ATCC 27853, *S. enteritidis* ATCC 13076, *S. typhi* ATCC 14028 and the yeast *C. albicans* ATCC 90028, proved to be resistant to all methanolic bee pollen extracts. The antibiotic used in the experiment, Streptomycin, was active for the bacteria *E. coli, P. aeruginosa, S. enteridis* and *S. typhi* (data not shown).

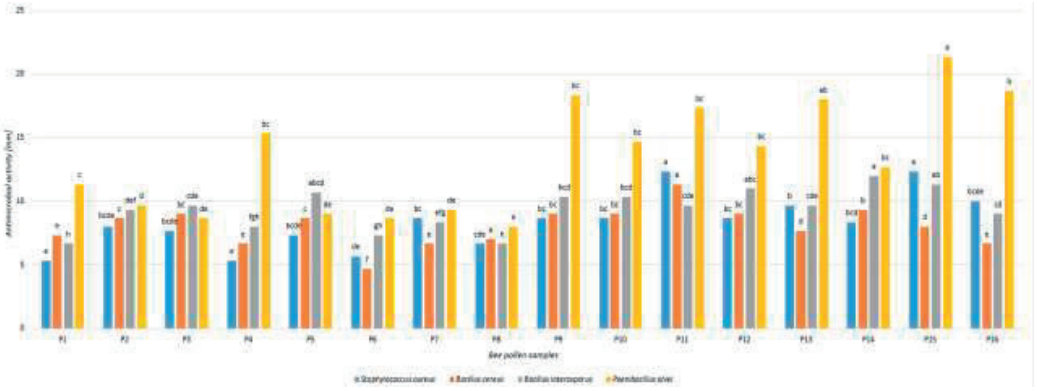

Figure 2. Antimicrobial activity of bee collected pollen samples. Results are given as the mean ± SD (n = 3). Values with different letters (a - h) are significantly different ($p < 0.05$), using ANOVA Tukey's multiple-comparison test. P1 - P16, samples of multifloral pollen

The diameter of the inhibition zones of the *S. aureus* strain falls within the limits of 5.33 ± 0.58 mm for samples P1 and P4 (both samples belonging to the family Rosaceae: P1 - *Crataegus monogyna*, P4 - *Malus domestica*) and 12.33 ± 0.58 mm determined for samples P11 (Rosaceae - *Rosa canina*) and P15 (Fabaceae - *Salix* sp.). The weakest action on *B.*

cereus strain was sample P6 sample (Rosaceae - *Prunus* sp.) with 4.67 ± 0.58 mm and the most intense action was noticed in sample P11 (Rosaceae - *Rosa canina*), 11.33 ± 0.58mm.

The *B. laterosporus* strain showed a minimum inhibition of 6.67 ± 0.58 mm determined by extracts from samples P1 (*Crataegus monogyna*) and P8 (*Taraxacum officinale*) and the maximum inhibition was determined by sample P14 (*Calluna vulgaris*) followed by P15 (*Salix* sp.).

Regarding *P. alvei* strain, the greatest action was noticed for sample P8 (*Taraxacum officinale*) and P15 (*Salix* sp.) with and an inhibition zone of 8.00 ± 0.00 mm and 21.33 ± 0.58 mm, respectively.

Carpes et al. (2007) used ethanolic extract from two pollen samples from Parana and Alagoas, with alcohol concentrations between 40-90%. The antibacterial activity differs both in terms of the concentration of the solvent and the sample used. Parana pollen showed no activity on *B. cereus* and *S. aureus* strains, but showed a 2.5 mm inhibition zone on the *P. aeruginosa* strain. Comparatively, the pollen from Alagoas showed an activity of 3 mm on *Stafilococcus aureus*, 0.5 mm on *B. cereus* and 1.00 mm on *P. aeruginosa*.

Abouda et al., 2011, studied various pollen samples from Morocco using extracts in DMSO. In their study, 14 pathogenic Gram-positive and Gram-negative bacterial strains showed resistance to 5 different types of antibiotic, but were sensitive to pollen extracts. This could be an interesting approach to control multiple microorganisms in the medical field as possible. As microorganisms, such as gram positive bacteria tend to develop resistance to certain antibiotics, our study showed that BCP proves to be an alternative product in this aspect. Furthermore, Velásquez et al. (2017) showed that there is a strong correlation between the chemical properties of different bee pollens and plant families.

Serial dilution method

The method of successive dilutions was tested on bacterial strains of *S. aureus*, *B. cereus*, *B. laterosporus*, and the obtained results are presented in Table 1. The presence of the microorganism in the well can be observed in samples P1, P4 - P6, P8 starting with the 1/8 dilution for the *S. aureus* strain, which means that starting from this dilution the methanolic extract no longer has an antimicrobial effect.

Regarding the *B. cereus* strain, the lack of antibacterial effect also starts from the 1/8 dilution for samples P1, P4, P6-8 and P16.

The same was noticed for *B. laterosporus* strain, with the exception of methanolic extracts from samples P1, P6 and P8.

Table 1. Minimum inhibitory concentration (MIC) of bee pollen methanolic extract

Sample	Strains tested														
	Staphylococcus aureus					*Bacillus cereus*					*Bacillus laterosporus*				
	Extract concentration tested														
	1/2	**1/4**	**1/8**	**1/16**	**1/32**	**1/2**	**1/4**	**1/8**	**1/16**	**1/32**	**1/2**	**1/4**	**1/8**	**1/16**	**1/32**
P1	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+
P2	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+
P3	-	-	-	+	+	-	-	-	+	+	-	-	-		+
P4	-	-	+	+	+	-	-	+	+	+	-	-	-	+	+
P5	-	-	+	+	+	-	-	-	+	+	-	-	-	-	+
P6	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+
P7	-	-	-	+	+	-	-	+	+	+	-	-	-	+	+
P8	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+
P9	-	-	-	+	+	-	-	-	+	+	-	-	-	-	+
P10	-	-	-	+	+	-	-	-	+	+	-	-	-	-	+
P11	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+
P12	-	-	-	+	+	-	-	-	+	+	-	-	-	-	+
P13	-	-	-	-	+	-	-	-	+	+	-	-	-	-	+
P14	-	-	-	+	+	-	-	-	+	+	-	-	-	-	-
P15	-	-	-	-	-	-	-	-	+	+	-	-	-	-	+
P16	-	-	-	-	+	-	-	+	+	+	-	-	-	+	+

+ well in which the tested microorganism was present; - well in which the tested microorganism was absent

Studies conducted by Abouda et al. (2011), Graikou et al. (2011), Pascoal et al. (2014) on antibacterial activity and implicitly the minimum inhibitory concentration of different types of bee pollen (Morocco, Greece, Portugal) in different solvents (methanol, ethanol, DMSO) had different results regarding MICs. This means that an important role in the control of the biological action of pollen extracts is played by both the botanical and geographical origin of the sample and the solvents used in the extractions.

Research has also shown that reference strains are much more sensitive than those isolated from biological fluids (Pascoal et al., 2014).

Statistical analysis

On the basis of the PCA a good discrimination of the BCP samples was achieved (the first two principal components explaining 89% of the data variance). In Figure 3 it can be observed that the botanical origin of pollen samples was correlated with the total phenolic content and antimicrobial activity. Samples P6, P11 and P15 had a very distinctive pattern, whereas samples P2, P3, P5 and P7 showed pattern similarities. The same was noticed for samples P10 and P12, respectively.

The variables close to the central axis (zero value) have similar concentrations in all BCP samples.

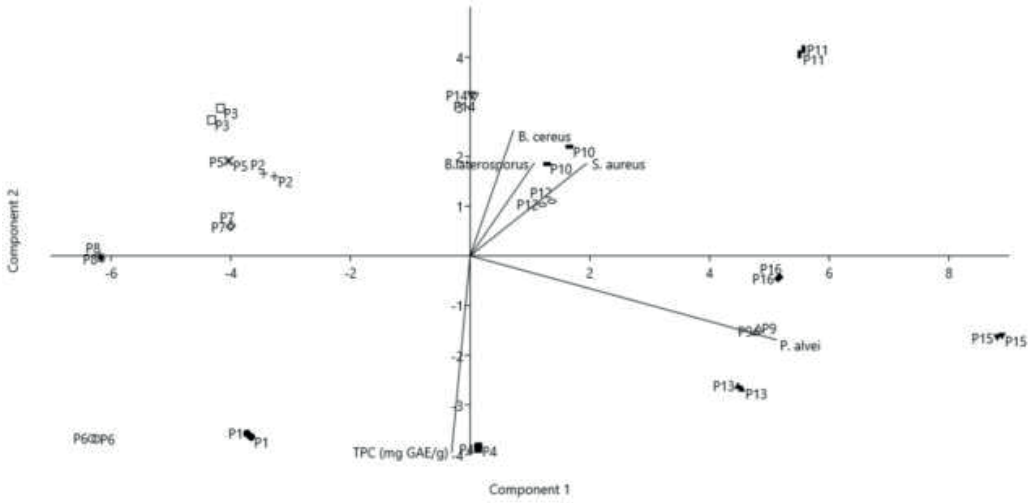

Figure 3. Principal component analysis biplot of the bee collected pollen samples. Two dimensions together explained 89% of the variance data

Thus, the total phenolic content and the antimicrobial activity against *P. alvei* significantly contribute to the discrimination between samples. In Figure 3, from left to right, the first group is formed by samples predominant in Rosaceae (P1, P4, P6 and P9), Brassicaeae (P13) and Fabaceae (P16) displaying significantly higher phenolic content compared to the other samples. In the second quadrant, sample P15 (Salicaceae - *Salix* sp.) possesses the highest antimicrobial activity against *P. alvei*, followed by samples P9, P13 and P16. The third quadrant highlights the antimicrobial activity against *S. aureus*, *B. cereus* and *B. laterosporus*. Samples P11 (Rosaceae - *Rosa canina*) and P15 (Salicaceae - *Salix* sp.) had the strongest antimicrobial activity against *S. aureus* and *B. cereus*, respectively. Sample P14 (Ericaceae - *Calluna vulgaris*) showed the highest inhibition zone against *B. laterosporus*. In the last quadrant, samples P2, P3, P5 and P7 showed pattern similarities, displaying the lowest phenolic content, as well as minimum inhibition zones in *S. aureus*, *B. cereus* and *B. laterosporus.* Comparatively, these samples had a moderate inhibitory activity against *P. alvei*.

CONCLUSIONS

Our study confirms that bee collected pollen is a rich source of phenolics, especially the

samples rich in Rosaceae, Brassicaeae and Salicaceae family, respectively. Furthermore, BCP possesses significant antimicrobial activity against gram positive bacteria, especially *S. aureus*, *B. cereus*, *B. laterosporus* and *P. alvei.* These findings are in accordance with previous reported researches which emphasized the importance of bee collected pollen as a functional food rich in nutrients and bioactive compounds.

ACKNOWLEDGEMENTS

This research work was financed from the project 26.526/07.12.2017.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

RESEARCH ON THE QUANTITATIVE AND QUALITATIVE EVOLUTION OF SHEEP'S MILK DURING THE LACTATION PERIOD

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Abstract

Sheep's milk is a very important food for human nutrition and is also an important raw material for a whole series of dairy products. The purpose of the current paper is to investigate the milk's quantitative and qualitative variations, during the lactation period in sheep. Milk samples were collected and some parameters, such as: quantity, density, percentage of fat, protein substances and lactose were monitored every month of lactation. The obtained results (compared to the 2nd month) showed significant increase (P<0,05) in the case of milk production for the 3rd month (with 10.9%) and for the 4th month (with 9.95%). Significant increase was also obtained in the case of milk density for the 4th month (with 0.29%), for the 5th month (with 0.48%) and for the 6th month (with 0.77%). Concerning the percentage of fat, it also showed increase for the 5th month (with 9.45%) and for the 6th month (with 24.32%). Simultaneous, significant decreases (P<0.05) were found in the case of milk production in the 5th month (with 10.6%) and in the 6th month (with 30.13), as well as in the percentage of lactose in the 5th month (with 4.79%) and in the 6th month (13.73%).

Key words*: fat, lactose, milk, sheep.*

INTRODUCTION

Sheep farming in our country is a traditional economic activity that has been a source of food and raw materials for the needs of the rural population, but also for trade (Teodorescu et al., 2013; Popica et al., 2014).

The diversity of dairy products and their distinguished biological value (Savu et al., 2002) made the sheep to be appreciated and, for these reasons, the breeders pay special attention.

Milk is a very balanced food, which is why a rational diet must necessarily include it beside other dairy products, that should be consumed daily (Vidu et al., 2014; Tăpăloagă et al., 2016; Oprea et al., 2019; Petcu et al., 2020; Oprea et al., 2020).

Due to its rich and varied chemical composition, milk provides most of the substances needed for living tissues and also for the maintenance of metabolic processes that take place in the body (Savu et al., 2002; Cotor et al., 2012; Oprea et al., 2019).

Sheep's milk is a major food for human nutrition and is also an important raw material for a whole series of dairy products, extremely appreciated in our country (Savu et al., 2002).

In order to obtain a quality milk, special attention must be paid to the mammary gland. Its development must be carefully monitored by the breeders, as the optimal production in terms of quantity and quality is known to be obtained only in healthy conditions (Tăpăloagă et al., 2018; Ghiță et al., 2019). Moreover, particular attention must also be paid to food safety, the manufacture, packaging and storage of final dairy products (Petcu, 2006; Petcu et al., 2014; Visoescu et al., 2015).

Studying the literature, we found many data on the chemical composition of sheep's milk (with important repercussions on the quality of dairy

products), but we did not find data on its quantitative and qualitative evolution, so this is the main reason for initiating the current research (Antunac et al., 2001; Lujerdean et al., 2008; Ghiță, 2010).
The aim of this research is to study how quantity and quality of sheep's milk change, during a lactation period.

MATERIALS AND METHODS

The biological material was represented by a group of 6 Ţurcană breed sheep, animals that were individualized and benefited from usual maintenance conditions (they were kept in the shelter at night and ate concentrates, and during the day they were taken to pasture, where they consumed plants from spontaneous flora).
The milk was collected every first day of the lactation from some certain months, in order to carry out this study. So, the data regarding the quantity and the quality of sheep's milk was obtained at the beginning of the following months of lactation: the 2nd (II), 3rd (III), 4th (IV), 5th (V) and 6th (VI) months of lactation.
The parameters followed were: the amount of milk, the density, the percentage of fat, the percentage of protein and the percentage of lactose.
The milk samples obtained were rapidly refrigerated (at a temperature of 4°C) and were immediately transported to the laboratory for processing.
The determination of the amount of milk was assessed individually for each animal, noting that the milking was done manually and the resulting amount of milk was measured with a graduated cylinder.
The milk density was determined with the lactodensimeter.
The determination of the percentage of protein substances was achieved by the titration method.
In the current study **the percentage of lactose** was determined using the potassium ferricyanide method (Savu et al., 2002).

RESULTS AND DISCUSSIONS

The obtained results are presented in a centralized manner in Table 1. The variation of the monitored parameters is presented in the form of graphs.
The comparisons on the statistical relevance of the differences between the ranges of values obtained were processed using the t-Student test.
Analyzing the data presented in Table 1, it is observed that the highest milk production was recorded in the 3rd (III) month of lactation (average production 1318.5 ml of milk) and in the 4th (IV) month of lactation (average production 1306.6 ml of milk).
It is also noted that in the 5th (V) month of lactation (average production 1062.1 ml of milk) and in the 6th (VI) month of lactation (average production 830.2 ml of milk), milk production decreases (in the 6th month a sheep was already weaned). So, the lactation graph has a bell shape (Figure 1), a fact also reported in the literature consulted (Antunac et al., 2001; Lujerdean et al., 2008; Cotor et al., 2012).

Table 1. The values of the parameters analyzed during the lactation period

Lactation month	Milk amount (ml)	Density	Fat (%)	Protein (%)	Lactose (%)
II	1188.3	1.034	7.4	5.10	4.44
III	1318.5*	1.035	7.13	5.18	4.52*
IV	1306.6*	1.037*	7.28	5.38*	4.47
V	1062.1*	1.039*	8.10*	5.56*	4.23*
VI	830.2*	1.042*	9.20*	6.05*	4.12*

*P<0.05

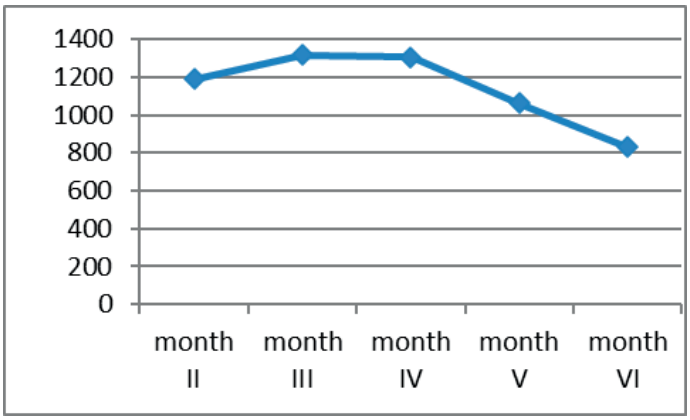

Figure 1. The variation of the average milk production, during the lactation period

As a consequence of the statistical analysis, the obtained results indicate a significant increase ($P<0.05$) of milk production in month III (by 10.9%) and IV (by 9.95%), compared to month II.

There is also a significant decrease ($P<0.05$) in milk production in months V (by 10.6%) and VI (by 30.13%), compared to the 2nd (II) month, when there was maximum amount of milk.

Results and discussions regarding the milk density

The highest value of milk density was recorded in the 6th (VI) month (end of lactation) and the lowest value was recorded in the 2nd (II) month (beginning of lactation), while in months III, IV and V the value of density was between the two limits, giving a graph with the appearance of an ascending slope (Figure 2).

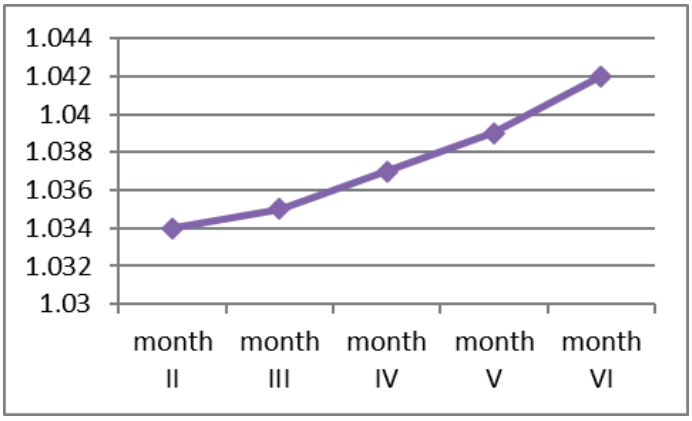

Figure 2. The variation of the average milk density, during the lactation period

The results indicate significant increases ($P<0.05$) of milk density in the 6th (VI) month (0.77%), in the 5th (V) month (0.48%) and in the 4th (IV) month (0.29%) compared to the 2nd (II) month.

In the 3rd (III) month there was an insignificant increase ($P>0.05$) by 0.09%, compared to the 2nd (II) month. These results can be explained by the increase in the percentage of dry matter in the feed, because in the middle and at the end of lactation (summer-autumn season) the proportion of coarse feed increased in the diet of the surveyed sheep.

Results and discussions regarding the percentage of milk fat

The percentage of fat had the highest value towards the end of lactation (9.20%). At the beginning of lactation (7.40%) and in the middle of lactation (7.28-8.10%) the percentage of fat was lower (Figure 3).

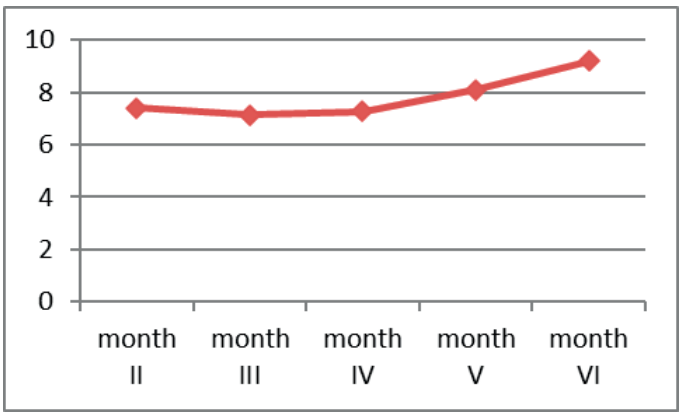

Figure 3. The variation of the percentage of fat in milk, during the lactation period

The obtained results indicate significant increases ($P<0.05$) of milk fat in the 6th (VI) month (24.32%) and in the 5th (V) month (9.45%) compared to the 2nd (II) month.

It is also observed that in the 3rd (III) and in the 4th (IV) months there were insignificant decreases ($P>0.05$) by 3.64% and 1.62%, compared to the 2nd (II) month.

An explanation of these results can be represented by the consumption of coarse feed; it is known that this type of food influences the concentration of fatty acids in milk (Cotor et al., 2015), as well as the size of fat globules in milk (Cotor et al., 2009).

Results and discussions regarding the percentage of milk protein

The highest percentage of protein is recorded at the end of lactation (6.05%), while in other periods of lactation the percentage of milk protein is less than 6% (Figure 4).

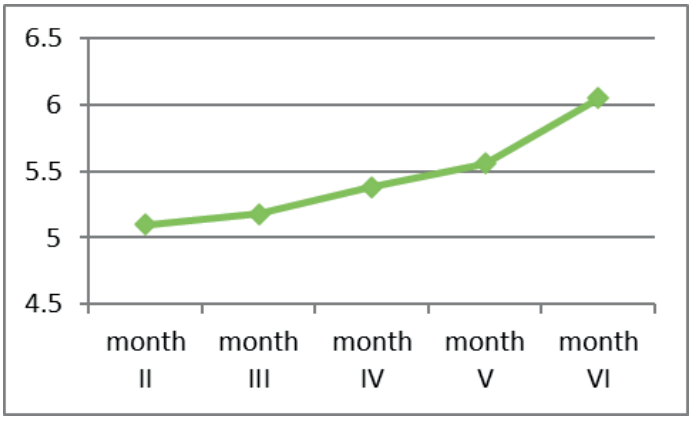

Figure 4. Milk protein percentage variation, during the lactation period

The results indicate significant increases ($P<0.05$) of milk protein in the 6th (VI) month (18.62%), in the 5th (V) month (9.01%) and in the 4th (IV) month (5.49%) compared to the 2nd

(II) month. In the 3rd (III) month there was an insignificant increase (P> 0.05) with 1.56%, compared to 2nd (II) month.
These results can be explained by the functional status of the mammary gland (Cotor et al., 2011) and by the diet in these periods.

Results and discussions regarding the milk lactose percentage.

The percentage of lactose varied only slightly and it has values between 4.52% and 4.12% (Figure 5).

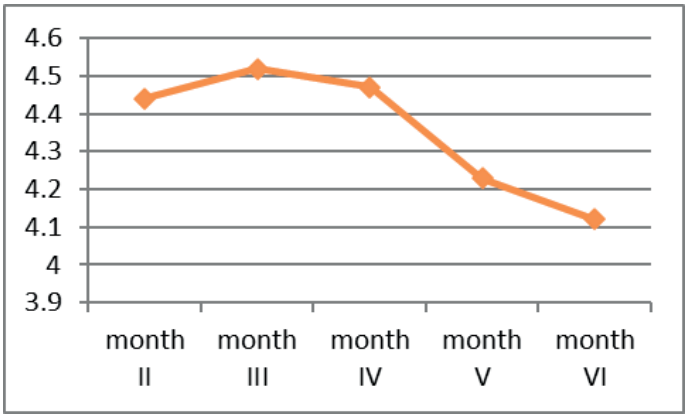

Figure 5. lactose percentage variation in milk, during the lactation period

The results indicate a significant increase (P<0.05) of lactose in the 3rd (III) month (1.8%) and in the 4th (IV) month there was an insignificant increase (P>0.05) with 0, 67%, compared to the 2nd (II) month.
Moreover, significant decreases (P <0.05) of lactose in milk were found in the 5th (V) month (4.79%) and 6th (VI) month (13.73%), compared to the 2nd (II) month. These variations are determined by the diet of the sheep correlated to the season.

CONCLUSIONS

Significant increases were found in the following parameters, compared to the 2nd (II) month of lactation: milk production, for the 3rd (III) month (by 10.9%) and the 4th (IV) month (by 9.95%), milk density for the 4th (IV) month (by 0.29%), for the 5th (V) month (by 0.48%) and for the 6th (VI) month (by 0.77%), fat percentage for the 5th (V) month (by 9.45%) and for the 6th (VI) month (by 24.32%), the percentage of protein for the 4th (IV) month (by 5.49%), for the 5th (V) month (by 9.01%) and for the 6th (VI) month (by 18.62%) and the percentage of lactose for the 3rd (III) month (by 1.8%);

Significant decreases were found (P <0.05) in the case of milk production in the 5th (V) month (by 10.6%) and in the 6th (VI) month (by 30.13%), and in the percentage of lactose in the 5th (V) month (by 4.79%) and in the 6th (VI) month (by 13.73%), compared to the 2nd (II) month of lactation.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

THE QUALITY EVALUATION OF SOME ASSORTMENTS OF CANNED PORK

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Abstract

The purpose of the study was the appreciation of the quality of some assortments of canned pork marketed in Romania, following the sensory, chemical (including energy value) and aesthetic characteristics. Three types of canned pork were taken in the study (pressed pork, pork in its own juice and hook meat). Were analysed 30 samples (coded A, B and C, ten samples for each assortments). Sensory characteristics were analysed by tasting, using the scoring scales method and the content of water, dry matter, proteins, lipids and salt was determined by standardized classical methods; the results obtained were compared with the values declared on the label. Following the sensory analysis, two products was included in the category of good and one in very good quality class. After laboratory analysis, the amount of protein was the closest (between 14.16% (for product A) and 15.06% (for product C), the amount of water varied between 61.05% (for product A) and 70.25% (for product C), that of lipids between 12.75% (for product C) and 22.80% (for product A) and that of salt between 2.05% (for product B) and 1.73% (for product A). The analysis showed very small differences in the chemical composition compared to the values indicated on the product label, but larger differences could be observed between the studied products, probably based on the different chemical composition of the raw material used.

Key words*: canned pork, energy value, lipids, proteins.*

INTRODUCTION

Food of animal origin is among those products that provide many important nutrients. The food industry employs numerous technologies which allow manufacturing of products with diversified shelf life. Canned products are characterized by a long shelf life, do not need to be kept at low temperature, and do not require special treatment during transport or distribution (Dave & Ghaly, 2011; Kapica & Weiss, 2012; Kowalska et al., 2020).

Canned pork is one of the widely sold meat products. The majority of canned meats are commercially sterilized and are processed to the point at which most microorganisms and their spores are killed. This capability allows to increase canned meat shelf life to a certain extent, provided it is kept sealed, but the product is markedly different from fresh meat, both chemically and physically in the course of time (Ferysiuk et al., 2020).

In the production of pork-type canned meat in its own gravy, the meat is grinded and then mixed, which could lead to the unification of the quality of meat batter; thereby, minimizing the risk of the development of point quality defects of the product (Florowski et al., 2017).

Sensory assessments of canned meat depend on other parameters, such as the quality of cans and raw materials, which need to be continuously examined to provide consumers safe products (Stojanović et al., 2021).

According to the 2006 Directive no. 52 and 2011 Order no. 1129 of European Commission (Commission Regulation EU), the maximum allowed limit of nitrite is 100 mg/kg in sterilized meat products.

In January 2016, upon the request of the European Commission, Food Chain Evaluation Consortium concluded in its report that an average dose of 80 ppm of added nitrite would be sufficient for sterilized meat products without significant effect on color, flavor and microbiological safety (Food Chain Evaluation Consortium, 2016).

The purpose of the study it was the appreciation of the quality of some assortments of canned pork marketed in Romania, following the sensory, chemical (including energy value) and aesthetic characteristics.

MATERIALS AND METHODS

Three types of canned pork (pressed pork, pork in its own juice and hook meat) randomly coded A, B and C, ten samples for each assortment) were taken in the study. Sensory characteristics were analyzed by tasting, using the scoring method; the lipid content was determined by the Soxhlet method, the protein one by the Kjeldahl method, and the moisture and the dry matter by the drying method in the oven (at 105°C).

The evaluation of the sensory quality of canned pork was carried out in a sensory analysis laboratory of USAMV Iasi by the participation of a group of twenty-four 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 and the *consistency* was analyzed on the outside and then in the products section with the touch analyzer, by chewing and visually. 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 organoleptic characteristic (SP 3196-83) was performed by comparing with scoring scales of 0-5 points, by 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 22 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 (according to STR 1125-85 Organoleptic characteristics for canned meat).

As a result, the arithmetic mean obtained from the score given by all tasters for each characteristic was taken. Examination of organoleptic characteristics specific canned pork followed: appearance, color, consistency, taste and smell.

RESULTS AND DISCUSSIONS

After the **sensory analysis** the total score determined for the products analyzed was between 19.86 and 20.31 points (good and very good quality class); however, the total average (20.01), includes all the analyzed canned pork in the category of very good products; two products were included in the good quality class, but with a very high score (19.90 and 19.86) close to the very good product that obtained 20.31 points (Table 2)

Table 1. Classification of the products in the appropriate quality class according to standards

Total average score	Quality class/grade obtained
20.1-22	Very good
17.6-20	Good
13.1-17.5	Satisfactorily
7.1-13	Unsatisfactory

Table 2. Total score obtained for the sensory analysis of the canned pork

Products	Total score	Qualifying
A	20.31	Very good
B	19.86	Good
C	19.90	Good
Average	20.02	Very good

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 (4.83) for product C and the lowest for consistency (3.78) for product B.

The summed score for canned pork was between 23.23 (for product A) and 22.07 points (for product B).
The three analysed products summed the following score for organoleptic characteristics: 14.22 for appearance, 13.92 for color, 13.86 for taste 13.33 for smell, and 12.75 for consistency, highlighting the highest value for appearance (14.22), color (13.92) and taste (13.86) for all studied canned pork.
On average was obtained the following values for all products: 4.74 for appearance, 4.25 for consistency, 4.64 for color, 4.44 for smell and 4.62 for taste.

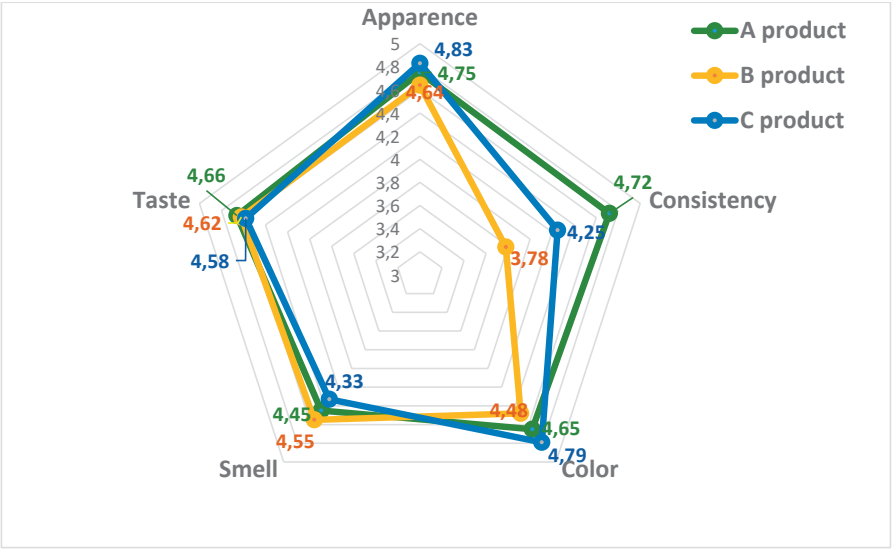

Figure 1. The average score of sensory characteristics determined by tasting for canned pork

The weighted average score obtained after tasting for the canned pork summed 27.72 points for taste, 16.00 for smell, 5.69 for appearance, 5.57 for color and 5.10 for consistency. The average score was 9.24 for taste, 5.33 for smell, 1.90 for appearance, 1.70 for consistency and 1.86 for color (Figure 2).

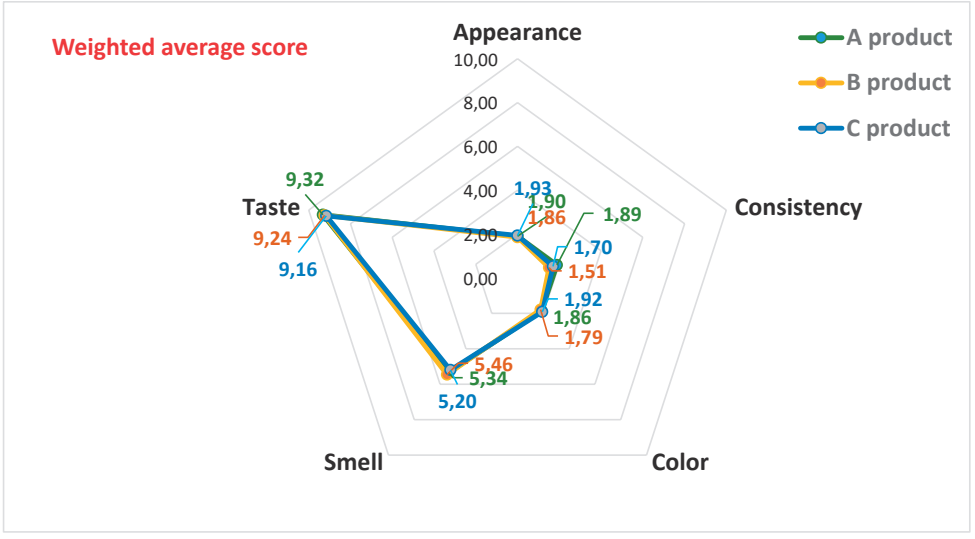

Figure 2. Weighted average score obtained after tasting for the canned pork

The average content determinate in laboratory of chemical composition for all canned pork taken in the study was: 65.91% for water, 17.41% for lipids, 14.29% for proteins, 1.87 for salt and 0.52 for carbohydrates, with *very* small differences between the values from the label (Table 3).

The energy value of analysed products was on average 221.02 kcal per 100 g (ranging between 177.18 – 262.39 kcal) on the label; the highest energy value (262.39 kcal per 100 g) was observed in the product which also contain the highest amount of lipids (23.01%).

Table 3. Chemical composition and energy value of the canned pork (values on the label and determined in laboratory)

Content / Product	Water	Lipids (%)		Proteins (%)		Salt (%)		Carbohydrates (%)		Energy			
										kcal/100g		kJ/100g	
	D*	L*	D*	L*	D*	L*	D*	L*	D*	L*	D*	L*	D*
A	61.05	23.01	22.8	14.31	14.16	1.78	1.73	0.21	0.26	262.39	267.13	1097.84	1117.65
B	66.43	17.5	16.68	14.44	13.66	2.00	2.05	2.06	1.18	223.50	213.35	928.0	892.65
	70.25	12.9	12.75	15.16	15.06	1.93	1.82	0.11	0.12	177.18	179.78	736.89	752.18
Average	**65.91**	**17.8**	**17.41**	**14.63**	**14.29**	**1.90**	**1.87**	**0.79**	**0.52**	**221.02**	**220.08**	920.91	920.83

**L - on the Label; *D - Determinated in laboratory.*

The largest variation was identified in case of lipids content (on the average, difference of 10.11 percentage points, between 12.9% and 23.01%), followed by water content (difference of 9.2 percentage points, between 70.25% and 61.05%).

For the protein content was observed relatively small differences of 0.85 percentage points (between 15.16% and 14.31%), for salt 0.22 percentage points, however for carbohydrates was identify differences of 1.95 percentage points (between 2.06% and 0.11%).

Product ingredients was:

-pork, salt, stabilizer (sodium diphosphate), antioxidant (ascorbic acid), dextrose, spices and spice extract, yeast extract, flavor enhancer (sodium monoglutamate), preservative (sodium nitrite) -***for product A***;

- pork, salt, onion, flavor enhancer (sodium monoglutamate), glucose syrup, flavors, stabilizer (sodium diphosphate), antioxidant (ascorbic acid, sodium ascorbate, citric acid), preservative (sodium nitrite)- ***for product B***;

- hook meat, salt, glucose syrup, antioxidant (sodium ascorbate), spices and spice extracts, *flavors*, stabilizer (sodium diphosphate), yeast extract, preservative (sodium nitrite) –***for product C.***

All three products contain sodium nitrite as a preservative and two of them contain sodium monoglutamate as a flavor enhancer. Unfortunately, a discovery the carcinogenic N-nitrosamines (NA) in fried bacon made in the 1971 raised concerns about the safety of nitrite use which remains unanswered to date (Sindelar et al., 2012). This led to a wide interest in the formation of NA in meat products and their influence on human health. NA can be generated during the usual processes applied to the products at home (e.g., cooking, frying), or in the products formed during the production process, or in the gastrointestinal environment through endogenous reactions. A common factor involved in the generation of NA is the reaction between a secondary amine and a nitrosating agent (Riviera et al., 2019; FAO, 2017). Moreover, a very high intake of nitrite can also lead to methemoglobinemia, a condition in which nitrite binds to hemoglobin and impairs the oxygen transport to cells (Gassara, 2016).

The current directive 2006/52/EC and the Regulation No. 1333/2008 state that the maximum amount of nitrite that may be added to the sterilized meat products is 100 mg/kg (Ferysiuk & Wojciak, 2020).

CONCLUSIONS

The canned pork studied have on average a very good score on a sensory point of view (at lower limit, 20.02 points), rated for five categories of characteristics: two of them obtaining over 19.8 points (19.86 and 19.90), and one product being very close to this (20.31 points). two products was included in the category of good and one in very good quality class. After laboratory analysis, the amount of protein was the closest (between 14.16% (for product A) and 15.06% (for product C), the amount of water varied between 61.05% (for product A) and 70.25% (for product C), that of

lipids between 12.75% (for product C) and 22.80% (for product A) and that of salt between 2.05% (for product B) and 1.73% (for product A).

The analysis showed very small differences in the chemical composition compared to the values indicated on the product label, but larger differences could be observed between the studied products, probably based on the different chemical composition of the raw material used.

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***STR 1125*-85 *Organoleptic characteristics for canned meat*

Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

ON THE INCLUSION OF SODIUM BENZOATE (E 211) AS ANTISEPTIC FOOD ADDITIVE IN SOFT DRINKS AND FISH PRODUCTS

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Abstract

Six products belonging in two food categories (carbonated soft drinks and fish products) have been investigated in laboratory via spectrophotocolorimetry to identify and quantify the usage of sodium benzoate as food additive with antiseptic-preservative purpose. The inclusion level of E-211 in the first group (carbonated soda) was 55-49% less than the maximum admitted inclusion level (AIL) (20 mg additive/100 g product, while the calculated daily intake through drinking a portion (500 ml) reached 32.7-34% out of the maximal admitted daily intake (MADI) for children and 11.25-17% of the MADI for adults. The concentration detected in the marinated fish was 25.1-35.1% lower than the AIL for this food category (200 mg/100 g). Calculus of the daily intake for a serving portion of marinated fish (env. 75 g) reached 64.9-74.6% of the MADI for children and 24.34-37.30% for adult consumers. Although the inclusion rates were below the maximal admitted limits, if we cumulate the potential intake of the sodium benzoate from these two food sources with other food preferred by children (sweet treats), the daily intake dose for this additive present becomes alarming and could endanger the health of young age consumers.

Key words*: daily intake, inclusion level, marinated fish*, *soda, sodium benzoate.*

INTRODUCTION

The most numerous group among additives that slow down food spoilage is represented by the antiseptic ones, commonly known as preservatives. These are either natural or synthetic chemical compounds added to food to restrict as much as possible the biological processes that take place in the product, e.g. the development of microflora and pathogenic microbes, and the effects of enzymes that affect food freshness and quality (Banu, 2010). In food products, preservatives change the permeability of cytoplasmic membranes or cell walls, damage the genetic system, and deactivate some enzymes (Pundir et al., 2011). Food is preserved using antiseptics or antibiotics (Luck & Jager, 1995). The former ones are synthetically produced simple compounds that often have natural correlates, and they make up no more than 0.2% of the product. Antibiotics, or substances produced by microorganisms, were also used in very small, yet effective, doses but they were eventually cancelled, due to antibioresistance installation in both foodborne pathogens and in consumers (Cebrián et al., 2026; Liao et al., 2020; Wales et al., 2015). The effectiveness of preservatives depends primarily on their effect on a specific type of microorganism, which is why it is vital to select the appropriate preservative based on the microbes found in the product (bacteria, mold, or yeast) (Ricke et al., 2005). Other factors that determine the effectiveness of preservatives include the pH value (a low pH is desirable), temperature, the addition of other substances, and the chemical composition of the product. Preservatives constitute an alternative to physical and biological product freshness stabilization methods, such as drying, pickling, sterilizing, freezing, cooling, and thickening. Consumer objections concerning the widespread use of chemical preservatives and their effects on human health have motivated producers to develop new food preservation procedures. These include irradiation, packaging, and storing products in a modified atmosphere, using aseptic technology (Yousef et al., 2012), along with newer biotechnological methods, as the usage of beneficial bacteria (Gao et al., 2019) or bacteriophage viruses capable to selectively

destroy the foodborne pathogens only (Bai et al., 2016). Products that are most commonly preserved include ready-made dishes and sauces, meat and fish products, fizzy drinks, and ready-made deserts (Millstone et al., 2003; Monneuse et al., 1997).

Other substances used as preservatives are acids and acidity regulators. These substances lower the pH level and slow down the growth of enzymes and yeasts, which hampers the development of fermentations and unwanted microbiota species (Stratford et al., 2013). They are used mainly in the production of marinades. Specific mixtures of acetic acid, salt and sugar are used to preserve and equally provide flavor to pickled vegetables (Komitopoulou et al., 2011). However, for a specific acid or acidity regulator to fulfil its role as a preservative, it needs to be added in highly concentrated form, but acetic acid, for instance, can irritate mucous membranes when its concentration exceeds 3% (A.O.A.C., 1990). Acids and acidity regulators are also used to enhance flavor (usually in fruit or vegetable products, or beverages, to bring out their sour taste) or to facilitate gelatinization and frothing during food processing (Multon, 1992; Wibertmann, 2000).

A Report of European Food Safety Authority from 2015 (EFSA, 2015) says that the most commonly used preservatives and antioxidants are sorbic acid and its salts (E200-203), benzoic acid and its salts (E210-213), sulfur dioxide (E220), sodium nitrite (E250), lactic acid (E270), citric acid (E330) and tocopherols (E306).

Other studies (Ratusz et al., 2013) demonstrate that mayonnaises and mustards are the fourth most often preserved product group, with ready-made concentrates ranking seventh.

The food products investigated within this study belonged to two groups in whose composition the benzoic acid and its salts can be included. The additives in this group, coded by the E numbers E210-E213 in the Codex Alimentarius catalogue (FAO, 2011), are known for inducing some adverse effects in consumers' health. The oral and/or dermal exposure to benzoic acid (Clemmensen et al., 1982) and to sodium benzoate could produce rush, asthma, rhinitis (Kumari et al., 2019; Scheman et al., 2012) or even allergic reactions leading to sudden anaphylactic shock in certain highly sensitive consumers (Aerts et al., 2020; del Olmo et al., 2017).

Experimental data, issued from laboratory analysis on the investigated products, served to estimate the daily ingested intake for sodium benzoate, in relation with the food category, with the consumer type (age, gender, body weight). All the data was interpreted in relation with the on-force regulations on the usage of sodium benzoate as antiseptic (preservative) food additive (EFSA, 2016; FAO, 2011).

MATERIALS AND METHODS

There have been studied two groups of food, in whose composition the usage of sodium benzoate as antiseptic additive is allowed at certain legal levels: "Carbonated soda" (maximum inclusion level of 20 mg/100 g edible portion) and "Marinated fish" (maximum inclusion level 200 mg per 100 g edible portion). Out of the first category (sauses), three brands of soda, have been investigated (coded Carbo Soda A, Carbo Soda B, Carbo Soda C). Out of the second category, three commercial products of "Marinated fish" type have been investigated (coded Marinade A, Marinade B, Marinade C).

The analytical method was derived from the A.O.A.C. 960.38 and 980.17 methods [1, 2] and has as principle the Beer's laws.

Equipment: UV-VIS VWR UV-6300PC (double beam, reading wavelength spectrum: 190-1100 ± 0.3 nm); quartz cuvettes; laboratory glassware (flasks of 150 ml, 100 ml balloons, 0.5, 1 and 10 ml pipettes).

Reagents: sodium benzoate 0.2% solution; bi-distilled and ultra-purified water.

Calibration curves: 6 successive diluted solutions of sodium benzoate are prepared (1; 2; 3; 4; 5; 6 ml sodium benzoate 0.2% solution added in 100 ml bi-distilled and ultra-purified water). Out of each dilution, there were taken 5 ml and were added to the measuring cuvettes. The blank sample cuvette is filled with 5 ml bi-distilled and ultra-purified water only. The successively diluted solutions, as well as the blank sample, were read in spectrophotometer between 200-300 nm wavelengths. The values read at 225 nm (wavelength at which the sodium benzoate exerts absorbance of the

photonic beam) were subtracted from the value read for the blank sample, resulting the quantitative values corresponding to 0.1-0.6 mg sodium benzoate.

Working procedure: 20 g (mashed) or 20 ml for each food product have been sampled and introduced into a 100 ml balloon. There were added 80ml bi-distilled and ultra-purified water to reach the whole balloon capacity. The balloons were steered then quantitatively filtered in 150 ml flasks. From the filtrate, 5 ml have been taken and pipetted into the measuring cuvettes. Those were scanned at 200-300 nm wavelengths, observing the peak readings for 225 nm. The readings were expressed as deviations from blank sample reading. Hence every cuvette contains a dilution equivalent of 1 g or 1 ml sample, each point of 0.1 mg on the calibration curve represents 0.01% sodium benzoate. Ten reading replicates have been run for each analyzed product.

The acquired data have been statistically interpreted, computing the main statistical descriptors (mean, standard mean error and variation coefficient). The means have been compared with the maximum tolerated limits of sodium benzoate inclusion in food and relative differences were also calculated. Starting from the average obtained values, the ADI (average daily intake) of E-211 were calculated, in relation with the legal allowance and with the type of consumer (child - 30 kg body weight, adult woman - 60 kg body weight, adult man - 80 kg body weight). When ADI was calculated, the size of consumed portions was considered in accordance with every product specificity and consumption habits: 500 ml for carbonated soda, 75 g of marinate fish.

RESULTS AND DISCUSSIONS

The data on the occurrence and concentration of sodium benzoate in the analyzed soda carbonate are presented in Table 1. In the situation of CarboSoda A samples, the analytical values varied within the 6 - 12 mg sodium benzoate /100 ml, resulting a mean of 9 mg/100 l, which represented 45% of the maximal inclusion level (20 mg E-211/100 ml product). In the other analyzed products, there were identified levels of 8-12 mg/100 l CarboSoda B, resulting an average content of 9.8 mg sodium benzoate/100 l, respectively values of 8-12 mg/100 l CarboSoda C, with an average of 10.2 mg sodium benzoate /100 l.

Table 1. Average values of the sodium benzoate contents in the three food products in the "soft drinks" category

Product	Analytical values			MAIL* (mg/100 ml)	% of MAIL
	$\bar{X}$	$\pm s_{\bar{x}}$	CV%		
CarboSoda A	9	0.45	15.71	20	45
CarboSoda B	9.8	0.53	17.07	20	49
CarboSoda C	10.2	0.28	8.77	20	51

* MAIL = maximal allowed inclusion level (mg/100 ml product)

In order to estimate the daily intake of sodium benzoate, the carbonated soda portion was considered of 500 ml. The results are presented in Table 2. It resulted that compared with the maximal allowed intake level (5 mg E-211/kg body weight), a child drinking such a carbonated soda portion will ingest a daily dose of 1.5 mg/kg BW - 1.7 mg/kg BW, which means 30-34% of the maximal allowed daily intake. If such a product would be eaten by adults, we estimated a daily intake of 0.75 mg/kg BW - 0.85 mg/BW in women, respectively of 0.563-0.638 mg/kg BW in men, resulting proportions of 15-17% of the maximal allowed daily intake in women and 11.25-12.75% in men (Table 2)

Table 2. Calculation of daily ingested dose sodium benzoate (E-211) through the three food products from the category carbonated soda

Daily ingested dose, related to consumer type	Product		
	CarboSoda A	CarboSoda B	CarboSoda C
MADI (mg E211/kg body weight)	5	5	5
Child, 30 kg body weight (mg E211/kg body weight)	1.500	1.633	1.700
% of MADI	30.0	32.7	34.0
Adult, woman, 60 kg body weight (mg E211/kg body weight)	0.750	0.817	0.850
% of MADI	15.00	16.33	17.00
Adult, man, 80 kg body weight (mg E211/kg body weight)	0.563	0.613	0.638
% of MADI	11.25	12.25	12.75

MADI - Maximal allowed daily intake

Results of the analytical trials related to the marinated fish are presented in Table 3.

Compared to the legal limit of E-211 inclusion for the food category "Fish products, salted/marinated/dry" (200 mg/100 g), the analytical values oscillated between 128-142

mg/100 g in Marinade A samples, between 112-140 mg/100 g in Marinade B and between 138-160 mg/100 g in Marinade C samples. Detection of such concentrations led to various proportions of remanence in the three products, respectively of 67.5%, 64.9% and 74.6%, compared with the maximal admitted level (200 mg/100 g) (Table 3).

Table 3. Average values of sodium benzoate (E-211) content in the three products analysed from the group fish products, salted/marinated/dry

Product	Analytical values			MAIL* (mg/ 100 mg)	% of MAIL
	$\bar{X}$	$\pm s_{\bar{x}}$	CV%		
Marinade A	135.0	2.07	4.85	200	67.50
Marinade B	129.8	2.88	7.01	200	64.90
Marinade C	149.2	1.64	3.47	200	74.60

*MAIL = maximal allowed inclusion level (mg/100 g product)

Starting from these values and considering the size of an eaten portions of marinated fish of 75 g per day, the daily intake of sodium benzoate has been calculated (Table 4).

If such products would be consumed by children weighing 30 kg, the daily intake would reach 3.245-3.730 mg sodium benzoate per kg body weight (64.9-74.6% of the maximal allowed daily intake dosage, i.e. 5 mg preservative E-211/kg body weight). In adult consumers, the daily intake varied between 1.623-1.865 mg sodium benzoate/kg body weight in women (60 kg) or between 1.217-1.399 mg sodium benzoate/kg body weight in men (80 kg), resulting levels of 32.45-37.30% and 25.31-27.98% of the maximal allowed daily intake level in both analyzed genders (Table 4).

Table 4. Calculation of daily ingested dose of sodium benzoate (E-211) through the three products from the category fish products, salted/marinated/dry

Daily ingested dose, related to consumer type	Product		
	Marinade A	Marinade B	Marinade C
MADI (mg E211/kg body weight)	5	5	5
Child, 30 kg body weight (mg E211/kg body weight)	3.375	3.245	3.730
% of MADI	67.5	64.9	74.6
Adult, woman, 60 kg body weight (mg E211/kg body weight)	1.688	1.623	1.865
% of MADI	33.75	32.45	37.30
Adult, man, 80 kg body weight (mg E211/kg body weight)	1.266	1.217	1.399
% of MADI	25.31	24.34	27.98

MADI - Maximal allowed daily intake

Although in the investigated foods, the intake proportions, compared to the maximal allowed daily intake were lower, if one child would consume a portion from both products in the same day, the real daily intake would reach 5.3-6.2%. In adult consumer, the daily cumulative intake of sodium benzoate from the two food categories would reach 2-4% of the daily maximal admitted intake level.

In both consumption scenarios, there must be proceeded with caution when children nutritional habits are considered, due to the cumulative intake of such food additives and, in particular, of sodium benzoate, from many other food categories (Kim et al., 2017). It is known that E-211 is also used in sweet treats and fizzy drinks, frequently consumed by toddlers, school pupils and teenagers (Trasande et al., 2018). It is known that there are common food consumption patterns and preferences in children of such ages for products rich in antiseptic-preserving additives (fast-food products, sweets, snacks and sodas) (Bemrah et al., 2008; Berentzen et al., 2015; Mischek et al. 2012).

CONCLUSIONS

The inclusion level of E-211 in the first group (carbonated soda) was 55-49% less than the maximum admitted inclusion level (AIL) (20 mg additive/100g product, while the calculated daily intake through drinking a portion (500ml) reached 32.7-34% out of the maximal admitted daily intake (MADI) for children and 11.25-17% of the MADI for adults.

The concentration detected in the marinated fish was 25.1-35.1% lower than the AIL for this food category (200 mg/100 g). Calculus of the daily intake for a serving portion of marinated fish (env. 75 g) reached 64.9-74.6% of the MADI for children and 24.34-37.30% for adult consumers.

Although the inclusion rates were below the maximal admitted limits, if we cumulate the potential intake of the sodium benzoate from these two food sources with other food preferred by children (sweet treats), the daily intake dose for this additive present becomes alarming and could endanger the health of young age consumers.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

MICROBIOLOGICAL EXAMINATION OF TELEMEA CHEESE IN CONSUMPTION NETWORK

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Abstract

In order to prevent the appearance of consumer diseases, any food products need to respect bacteriological standards; to not contain pathogenic germs, and the saprophytes ones have to be under maximal norm limits. In this study there were harvested 30 samples of Telemea cheese, from two different production units, in two different counties. The samples were collected in two different seasons: summer and winter and were microbiological analysed, in order to determine the following parameters: coliform bacteria, E. coli, Coagulase-positive staphylococci, Salmonella, total number of yeasts and moulds. The result were statistical evaluated, establishing each microbiological index dynamic, depending on season.

Key words*: cheese, consumer, pathogenic germs, saprophyte germs.*

INTRODUCTION

Telemea cheese is a traditional product made from milk (Georgescu, 2000). The characteristics of this range of cheeses consist in: manufacturing technology, relatively high salt content and the fact that they are matured and preserved until consumption in brine (Costin, 2003). The assessment of the microbiological quality of cheeses is difficult, because of the technological microflora used in their manufacture. Microbiological control of cheeses involves the identification of pathogenic germs or the establishment of the causes of defects. (Bărzoi, 2002).

MATERIALS AND METHODS

The microbiological control was performed on a number of 30 Telemea cheese samples, collected from two processing locations (Ilfov County - P3 and Câmpulung Muscel - P5), in the warm and cold seasons.

The working procedure used consists in determining the following microbiological parameters: coliform bacteria, *E. coli*, *Salmonella*, *Staphylococcus* coagulase positive, Yeasts and molds.

The method to determine coliform bacteria involves insemination in BBLV, with fermentation tube and incubating at 37°C, 24-48 hours. Brilliant Green Bile Broth (BBLV) is a liquid medium used for the detection or confirmation of coliform bacteria in water and wastewater, foods, dairy products and other materials of sanitary importance. The development of some bacterial cultures with gas production in the fermentation tubes, consisting exclusively of Gram bacilli or coccobacilli, were considered positive reactions for coliform bacteria. The results were interpreted and the analysis continues with the determination of *E.coli* species. From the tubes tested positive for coliform bacteria, there were done inseminations in Petri dishes with Levine medium. The confirmation was performed in BBLV, tryptone water and slanted agar with incubation at 45°C, for 24 hours.

The work technique for *Salmonella* sp. isolation and identification, by horizontal method, assume the following steps:

- Stage I - Pre-enrichment in unselective liquid mediums.

The *Salmonella* bacteria may be in low number and often accompanied by a large number of

Enterobacteriaceae or other bacteria species. Pre-enrichment is necessary to discover the low number of *Salmonella* sp. or modified *Salmonella* sp.
Buffered peptone water is inoculated along with sample, then incubated at 37°C ± 10°C for 18 h ± 2 h.
- Stage II - Enriching in selective liquid mediums.
The Rappaport-Vassiliadis soybean medium (RVS broth) and tetrathionate/novobiocin Muller-Kauffmann broth (MKTTn broth) are inoculated with bacteria culture medium obtained in buffered peptone water. The RVS broth is incubated at 41.5°C ± 1°C for 24 hours ± 3 hours and the MKTTn broth at 37°C ± 1°C for 24 hours ± 3 hours.
- Stage III - Isolation and identification.
From the culture mediums obtained, two solid selective mediums are inoculated: xylose-lysine-dezoxycholate agar (XLD agar - Figure 1) and Rambach agar (Figure 2). They are incubated at 37°C ± 1°C and examined after 24 h ± 3 h.
- Stage IV - Identity confirmation.
Isolated colonies, presumed as *Salmonella* sp., are confirmed by biochemical and serological tests.
The detection of a large number of yeasts and molds in food products indicates the existence of inadequate hygienic conditions during food products obtaining and storage. (Apostu, 2006). The determination method involves performing decimal dilutions and inseminations on suitable mediums (Sabouraud agar with glucose and chloramphenicol). After 4-5 days of incubation, the formed colonies are counted.
Coagulase-positive staphylococci are microorganisms that form typical colonies on the surface of selective culture mediums. These microorganisms show a positive coagulase reaction, the coagulase production being a main index for enterotoxicity evaluation (Bărzoi, 2002). The determination method involves performing several steps: decimal dilutions and inseminations in Petri dishes with Chapman agar, incubation and developed colonies counting and performing the coagulase test.

RESULTS AND DISCUSSIONS

In Table 1, there are presented the results obtained after microbiologic examination of finite product, in P3 location, in warm season.

Table 1. Results obtained after microbiologic examination of finite product, in P3 - warm season.

No.	Coliform bacteria/g	Coagulase-positive staphylococci/g	Yeasts and molds/g
1	15	10	864
2	9.5	10	769
3	7.5	9	753
4	6.5	7	851
5	7.5	8	982
6	7.5	8	971
7	9.5	6	860
8	3	5	764
9	4	9	853
10	6.5	9	874
11	7.5	8	930
12	9.5	10	926
13	7.5	9	897
14	9.5	8	925
15	9.5	6	760
16	3	7	824
17	4	5	930
18	6.5	10	885
19	9.5	9	760
20	9.5	9	774
21	7.5	8	768
22	6.5	7	528
23	4.5	6	560
24	7.5	9	589
25	4	8	974
26	6.5	10	831
27	9.5	10	658
28	7.5	8	706
29	9.5	7	821
30	4.5	3	930

It can be observed that coliform bacteria had values between 3 and 15 germs/g, Coagulase-positive staphylococci had values from 3 to 10 germs/g and contamination with *E. coli* and *Salmonella* was absent. For yeasts and mold there was determined a number between 528 to 982 germs/g.
In Figure 1 there is presented the degree of contamination variation with coliform bacteria, for Telemea cheese, in warm season, in P3 location. This parameter it is a line which has a descending trend.

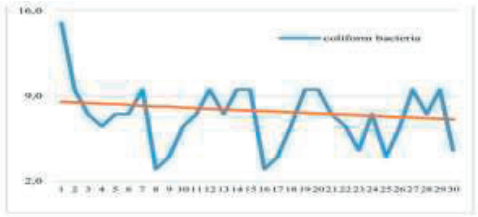

Figure 1. Dynamic of coliform bacteria contamination in Telemea cheese, in warm season, in P3 location

In Figure 2 there is presented the Coagulase-positive staphylococci fluctuation, observed in finite product during warm season. This parameter is also represented throw a line which has a descending trend.

Figure 2. Dynamic of Coagulase-positive staphylococci contamination in Telemea cheese, in warm season, in P3 location

In Figure 3 there is presented the Yeasts and mold fluctuation, observed in finite product during warm season. This parameter is represented also throw a line with a descendent trend, which means an improvement of the hygienic parameters provided in the legislation of dairy products

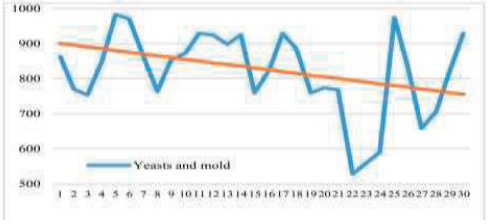

Figure 3. Dynamic of Yeasts and molds contamination in Telemea cheese, in warm season, in P3 location

In Table 2 there are presented the statistic analyses of main level and dispersion parameters for the analysed sample taken in P3 location, in warm season.

Table 2. Analyses of main level and dispersion parameters for the analysed sample taken in P3 location, in warm season

Parameter	n	$\bar{x}$	S^2	S	CV%
Coliform bacteria	30	7.33±0.46	6.60	2.57	35.05
Coagulase-positive staphylococci	30	7.93±0.32	3.10	1.76	22.18
Yeasts and molds	30	817.23±21.90	14399.90	120	14.68

The variability coefficient registered for coliform bacteria has the highest values (35.05%)

In Table 3, there are presented the results obtained after microbiologic examination of finite product, in P5 location, in warm season.

Table 3. Results obtained after microbiologic examination of finite product, in P5, warm season

No.	Coliform bacteria/g	Coagulase-positive staphylococci/g	Yeasts and molds/g
1	6.5	2	654
2	7.5	6	589
3	7.5	5	573
4	6.5	5	569
5	6.5	3	681
6	3.5	1	536
7	4	7	490
8	2.5	6	486
9	6.5	5	512
10	4	8	607
11	4	6	617
12	7.5	7	530
13	7.5	5	589
14	6.5	3	573
15	7.5	4	494
16	6.5	4	602
17	7.5	8	612
18	3.0	8	634
19	2.5	7	656
20	6.5	1	594
21	2.5	3	586
22	4	6	703
23	6.5	2	684
24	6.5	8	690
25	3.5	7	681
26	2.5	6	594
27	7.5	5	659
28	4.5	8	549
29	6.5	5	638
30	4	3	679

It can be observed that coliform bacteria had values between 2.5 and 7.5 germs/g, Coagulase-positive staphylococci had values from 1 to 8 germs/g and contamination with E.coli and Salmonella was absent. For yeasts

and mold there was determined a number between 486 to 690 germs/g.
In Figure 4 there is presented the degree of contamination variation with coliform bacteria, for Telemea cheese, in warm season, in P5 location. This parameter is represented by a line which has a descending trend.

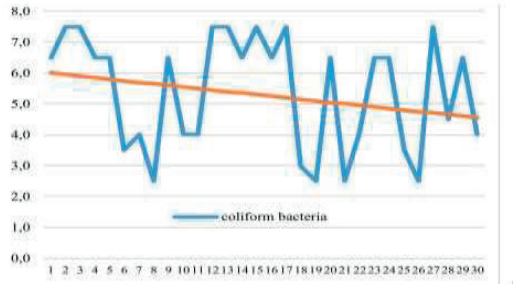

Figure 4. Dynamic of coliform bacteria contained in Telemea chese, in warm season, in P5 location

In Figure 5 there is presented the degree of contamination variation with Coagulase-positive staphylococci, for Telemea cheese, in warm season, in P5 location. This parameter is represented by a line which has an ascending trend.

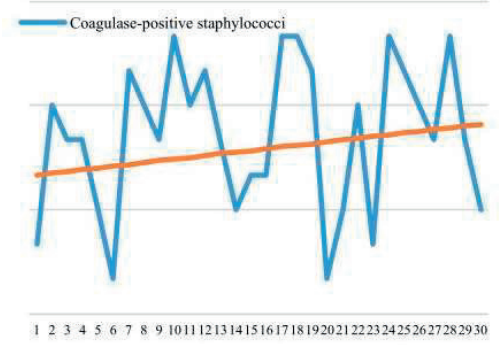

Figure 5. Dynamic of Coagulase-positive staphylococci contamination in Telemea cheese, in warm season, in P5 location

In Figure 6 there is presented the Yeasts and mold fluctuation, observed in finite product during warm season, in P5 location. This parameter is represented throw a line which has a slightly ascendent trend.

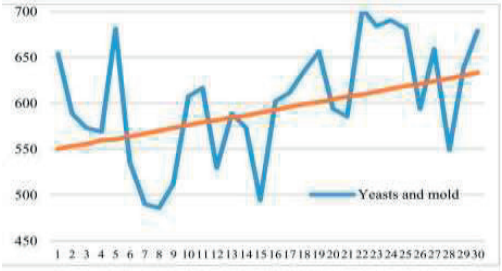

Figure 6. Dynamic of Yeasts and molds contamination in Telemea cheese, in warm season, in P5 location

In Table 4 there are presented the statistical analyses of main level and dispersion parameters for the analysed sample taken in P5 location, in warm season.

Table 4. Analyses of main level and dispersion parameters for the analysed sample taken in P5 location, in warm season

Parameter	n	$\bar{x}$	S^2	S	CV%
Coliform bacteria	30	5.4±0.33	3.45	1.85	34.42
Coagulase-positive staphylococci	30	5.13±0.39	4.62	2.14	41.79
Yeasts and molds	30	602.03±11.55	4007.76	63.30	10.51

The variability coefficient registered for Coagulase-positive staphylococci in warm season, P5 location, has the highest value (41.79%)
Testing the significance of the differences for Coliform bacteria in Telemea cheese, in warm season, it was found that between P3 and P5 locations, the differences are distinctly significant (Table 5). This is due to fact that in these locations the HCCP plan (Hazard analysis Critical Control Point Read) is implemented.

Table 5. Analyses of main level and dispersion parameters for the analysed sample taken in P5 location, in warm season

Parameter	**Calculated t**	**table t (t α)**			
		$t_{0.05}$	**$t_{0.01}$**	**$t_{0.001}$**	**$t_{0.1}$**
Coliform bacteria	3.33 **	2.00	**2.66**	3.46	1.29
Coagulase-positive staphylococci	5.52***				
Yeasts and molds	8.68***				

After testing the significance of the differences between P3 and P5 locations, in warm season, it was found that for Coagulase-positive staphylococci and for yeast and mold parameters, the differences are very significant.
In Table 6, there are presented the results obtained after microbiologic examination of finite product, in P3 location, in cold season.

Table 6. Results obtained after microbiologic examination of finite product, in P3 - cold season

No.	Coliform bacteria/g	Coagulase-positive staphylococci/g	Yeasts and molds/g
1	4	4	861
2	3	9	763
3	3	9	654
4	2.5	2	817
5	3.5	8	980
6	6.5	8	873
7	3.5	6	845
8	4	3	762
9	3.5	9	814
10	7.5	8	861
11	7.5	8	918
12	2.5	8	935
13	9.5	5	863
14	7.5	6	918
15	6.5	5	734
16	4	4	721
17	4.5	7	818
18	3	9	862
19	3	3	715
20	6.5	4	761
21	2.5	9	753
22	7.5	6	524
23	4	8	540
24	3	6	579
25	6.5	8	871
26	7.5	8	739
27	6.5	7	698
28	4	6	725
29	3	2	886
30	5.5	5	694

It can be observed that coliform bacteria had values between 2.5 and 9.5 germs/g, Coagulase-positive staphylococci had values from 2 to 9 germs/g and contamination with E.coli and Salmonella was absent. For yeasts and mold there was determined a number between 524 to 980 germs/g.
In figure 7 there is presented the degree of contamination variation with coliform bacteria, for Telemea cheese, in cold season, in P3 location. This parameter it is a line which has an ascending trend.

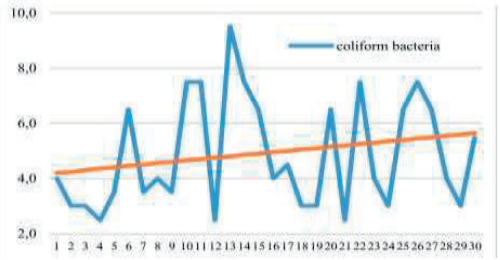

Figure 7. Dynamic of coliform bacteria contamination in Telemea cheese, in cold season, in P3 location

In Figure 8 there is presented the Coagulase-positive staphylococci fluctuation, observed in finite product during cold season. This parameter is represented throw a line which has a descending trend.

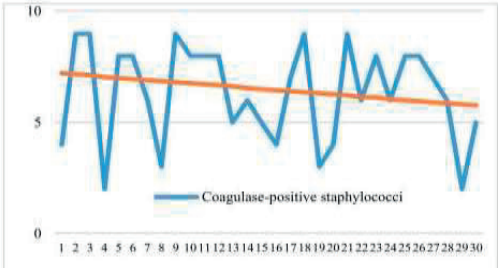

Figure 8. Dynamic of Coagulase-positive staphylococci contamination in Telemea cheese, in cold season, in P3 location

In Figure 9 there is presented the Yeasts and mold fluctuation, observed in finite product during cold season. This parameter is represented also throw a line with a descendent trend, which means an improvement of the hygienic parameters provided in the legislation of dairy products

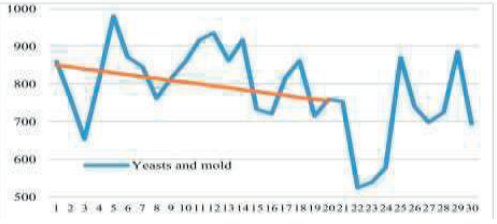

Figure 9. Dynamic of Yeasts and molds contamination in Telemea cheese, in cold season, in P3 location

In Table 7 there are presented the statistical analyses of main level and dispersion

parameters for the analyzed sample taken in P3 location, in cold season.

Table 7. Analyses of main level and dispersion parameters for the analyzed sample taken in P3 location, in cold season

Parameter	n	$\bar{x}$	S^2	S	CV%
Coliform bacteria	30	4.85±0.36	4.03	2.00	41.42
Coagulase-positive staphylococci	30	6.33±0.40	4.85	2.20	34.77
Yeasts and molds	30	782.8±20.75	12921.96	113.67	14.52

The variability coefficient registered for coliform bacteria has the highest values (41.42%).

In table 8, there are presented the results obtained after microbiologic examination of finite product, in P5 location, in cold season.

Table 8. Results obtained after microbiologic examination of finite product, in P5, cold season

No.	Coliform bacteria/g	Coagulase-positive staphylococci/g	Yeasts and molds/g
1	4	3	653
2	4.5	4	586
3	3.5	6	537
4	6.5	3	558
5	7.5	3	680
6	6.5	2	530
7	6.5	8	489
8	4	2	468
9	3.5	7	570
10	4	8	603
11	6.5	2	607
12	7.5	4	508
13	4	5	568
14	3.5	6	537
15	2.5	6	518
16	4	3	620
17	6.5	4	621
18	6.5	8	624
19	4	6	646
20	3.5	5	549
21	4	3	568
22	2.5	7	603
23	4	8	584
24	3	2	609
25	4	5	549
26	3.5	4	638
27	4	3	522
28	2.5	3	518
29	7.5	5	658
30	4	3	539

Coliform bacteria had values between 2.5 and 7.5 germs/g, Coagulase-positive staphylococci had values from 2 to 8 germs/g and contamination with E.coli and Salmonella was absent. For yeasts and mold there was determined a number between 468 to 680 germs/g.

In Figure 10 there is presented the degree of contamination variation with coliform bacteria, for Telemea cheese, in cold season, in P5 location. This parameter is represented by a line which has a descending trend, which means that this parameter was better monitored.

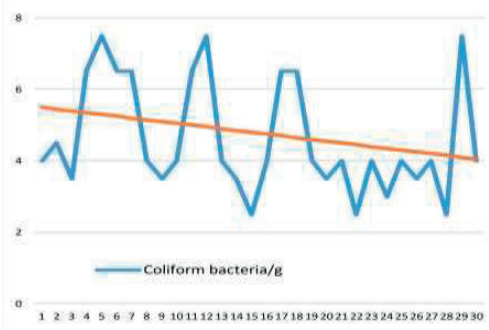

Figure 10. Dynamic of coliform bacteria contamination in Telemea chese, in cold season, in P5 location

In Figure 11 there is presented the degree of contamination variation with Coagulase-positive staphylococci, for Telemea cheese, in cold season, in P5 location. This parameter is represented by a line which present a relative constant values

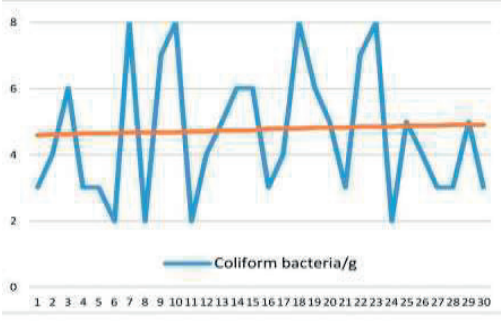

Figure 11. Dynamic of Coagulase-positive staphylococci contamination in Telemea cheese, in cold season, in P5 location

In Figure 12 there is presented the Yeasts and mold fluctuation, observed in finite product during cold season, in P5 location. This parameter is represented throw a line which also present a relative constant values.

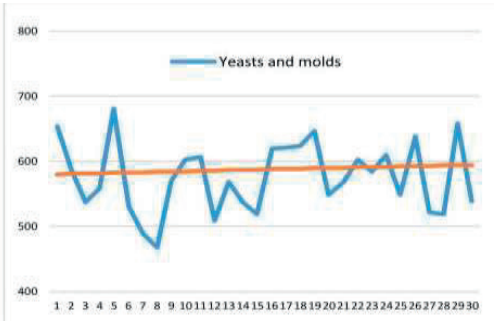

Figure 12. Dynamic of Yeasts and molds contamination in Telemea cheese, in cold season, in P5 location

In Table 9 there are presented the statistical analyses of main level and dispersion parameters for the analysed sample taken in P5 location, in cold season.

Table 9. Analyses of main level and dispersion parameters for the analysed sample taken in P5 location, in cold season

Parameter	n	$\bar{x}$	S^2	S	CV%
Coliform bacteria	30	4.6±0.28	2.5	1.58	34.42
Coagulase-positive staphylococci	30	4.6±0.36	3.97	1.99	43.32
Yeasts and molds	30	575.33±9.83	2900.37	53.85	9.36

The variability coefficient registered for Coagulase-positive staphylococci in P5 location, in cold season, has the highest value (43.32%)

Table 10 shows the differences between the values obtained in the locations where the Telemea cheese was analysed.

Table 10. Analyses of main level and dispersion parameters for the analysed sample taken in P5 location, in cold season

Parameter	**Calculated t**	**table t (t α)**			
		$t_{0.05}$	**$t_{0.01}$**	**$t_{0.001}$**	**$t_{0.1}$**
Coliform bacteria	0.53^{NS}	2.00	**2.66**	3.46	1.29
Coagulase-positive staphylococci	3.19**				
Yeasts and molds	9.03***				

After testing the significance of the differences between P3 and P5 locations, in cold season, it was found that for Coliform bacteria, differences are insignificant, for Coagulase-positive staphylococci, the differences are distinctly significant and for yeast and mold parameter the differences are very significant.

CONCLUSIONS

After testing the significance of the differences between P3 and P5 locations, in the summer season, a distinctly significant difference was found for coliform bacteria, and for Coagulase-positive staphylococci and Yeasts and molds the differences were very significant.

The determination of coliform bacteria in food provides information on the hygienic conditions in which the products were obtained, which is why it is recommended to review the existing regulations in the researched locations.

In cold season the differences between P3 and P5 locations, for coliform bacteria are insignificant.

Distinctly and very significant differences found between P3 and P5 locations, for Coagulase-positive staphylococci, and yeast and mold parameter underline the need to use the HACCP system, in order to eliminate the sources of contamination, in order to obtain healthy and safe products for consumption.

Following the obtained results, it can be said that location P3 has poorer hygiene conditions than location P5 where the HACCP system is implemented, which makes the dairy product, Telemea cheese, less wholesome.

For this reason, it is mandatory to urgently implement food quality assurance systems.

It is also necessary a training and qualification of the staff, through specialized courses, for the awareness and understanding of the quality concept and the importance of ensuring it for the health of consumers.

Food processing technologies will be less efficient if no increased attention is paid to laboratory analysis and expertise, inspection methods, staff training and improving the performance of investigative equipment according to current requirements. (Enache, 2005).

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

SHELF LIFE OF EGGS FROM HENS FED DIETS RICH IN POLYUNSATURATED FATTY ACIDS AND ANTIOXIDANTS UNDER THE EFFECT OF DIFFERENT STORAGE TIME AND TEMPERATURES

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Abstract

We investigated the effectiveness of diets enriched with natural sources of polyunsaturated fatty acids and antioxidants on shelf life, internal and external quality parameters of eggs in different storage time and temperature. The diets given were as follow: basal diet (C), a diet containing mixture of rapeseed and grapeseed meal (T1) and a diet containing mixture of flaxseed and sea buckthorn meal (T2). A total of 108 eggs were selected (36 eggs/ group) and were separated into 6 batches with 6 eggs in each. The experimental design was consisted of 3 storage periods (0, 14 and 28 days) and 2 storage temperatures (5°C, 21°C). Each egg was weighed and broken, and the physiochemical properties of eggs such as albumen, yolk and eggshell weight, eggshell thickness and breaking strength and the most important freshness parameters Haugh Unit (HU), albumen and yolk pH were determined using an Egg Analyzer TM, manufactured by Orka Technology Ltd. The egg, albumen, shell weight and HU significantly decreased with increasing storage time and temperature, especially for eggs stored at 21°C for 28 days from C group. The albumen and yolk pH significantly ($P<0.05$) increased with increasing storage time and temperature in all samples, but those from group C were significantly ($P<0.05$) higher compared with eggs from T1 and T2 groups. The interaction effects between the storage time and temperature were significant for all determined parameters, which can conclude that storage time and temperature are the major factors affecting egg quality, but temperature is a more sensitive determinant of egg quality deterioration compared with the storage period. Eggs from groups fed diets rich in polyunsaturated fatty acids and antioxidants significantly ($P<0.05$) delayed egg deterioration in both storage temperatures.

Key words*: egg quality, shelf life, storage time, temperature.*

INTRODUCTION

For many years the most important external and internal egg quality characteristics have been shown to be, and still are, egg weight, shell characteristics, yolk and albumen ratio and freshness parameters, given by albumen and yolk pH and Haugh Unit (HU) (Samli et al., 2005). All these parameters have a major impact on shelf life of eggs. According to Directive CE 13/2000 and CE 557/2007, regarding the marketing standards for eggs, defines shelf life as the period from the collection of the eggs to their consumption, during which time the product is in a satisfactory state of quality in terms of chemical, physical, microbiological and sensory attributes. Egg quality consists of different aspects, each of which can be related to internal or external egg quality. The internal egg quality relates in general to the quality of the albumen and yolk (Bamelis, 2003). Albumen quality is not only an important indicator for egg freshness, but it is also important for the egg breaking industry because albumen and yolk have different markets (Scott & Silversides, 2000). The quality of eggs including their weight, shell characteristics and internal components are affected by a wide range of factors of which the genotype, nutrition, age of hens, temperature, humidity, the presence of carbon dioxide (CO_2), and storage time are the most important factors in terms of maintaining egg quality. Storage time and temperature appear to be the most crucial factors affecting albumen quality and HU. It has been reported (Silversides & Budgell, 2004) that pH is a useful tool for describing the changes in albumen quality over time during storage. Albumen pH increases with the loss of CO_2 from the egg. An increase in pH has been reported by extending the storage time from 2

to 30 days (Abdel-Nour et al., 2011). Moreover, laying hens nutrition is an important factor for controlling egg internal and external components quality and can successfully enrich the egg in some minor components of interest for human nutrition. Some meals were investigated in many studies to assess their effect on egg quality of the laying hens as reported previously (Sărăcilă et al., 2017; Vlaicu et al., 2017a; Gheorghe et al., 2019; Turcu et al., 2019). Others (Lokaewmanee et al., 2014) reported that dietary plant extracts improved significantly the eggshell quality and breaking strength. Sharma et al. (2009) observed increased eggshell thickness (by 10.0%) and breaking strength (by 15.2%) in hens fed diet supplemented with herbal products. Panaite et al. (2019) reported that diet supplemented with sea buckthorn mixture, significantly ($P<0.05$) decreased albumen and yolk pH after 4-week storage, compared with control diet. Also, it was reported (Mridula et al., 2012) that the HU from hens fed 10% flaxseed was higher that control eggs, whereas in contrast Najib & Al-Yousef (2010), reported no effect on HU and yolk index at 10% flaxseed inclusion rate. Recently, Panaite et al. (2020) reported that a mixture of 10% rapeseed meal with 2.5% flaxseed meal had no effect on HU, but significantly ($P<0.05$) affected albumen pH. No impact on egg internal and external quality parameters were also reported in other studies (Bozkurt et al., 2012; Swiatkiewicz et al., 2013; Vlaicu et al., 2017b), when hens were fed enriched diets. Very few authors reported the results of enriched diets on shelf life of eggs under the effect of different storage temperatures in time to provide more evidence for preserving conditions of enriched egg, in order to extend the shelf life.

With this regard, the aim of this study was to investigate the effect of diets enriched with natural sources of polyunsaturated fatty acids and antioxidants, given by mixture of rapeseed with grapeseed meal and a mixture of flaxseed with sea buckthorn meal in laying hens, in order to put into evidence the evolution of their effect on shelf life, internal and external quality characteristics of eggs under the effect of different storage conditions, as time and temperature, for 28 days.

MATERIALS AND METHODS

Experimental design: The eggs used in this study were obtained from one hundred twenty, 50- to 56-week-old Tetra SL LL hens, which were included in a laying trial at the experimental facilities of the Department of Chemistry and Animal Nutrition Physiology from National Research-Development Institute for Animal Biology and Nutrition, Balotesti, Romania. The study was conducted on external and internal egg components of laying hens which were fed 6 weeks (42 days) with diets enriched with some natural sources of polyunsaturated fatty acids and antioxidants. The laying hens were housed in an experimental hall having identical conditions, equipped with Big Dutchman three-tier cages (2 hens/cage; 20 cages/group) dimensioned according to the sanitary-veterinary norms regarding the minimum standards for protection and handling of laying hens, monitored by a Viper Touch computer (temperature of 21°C to 23°C, humidity of 67 to 70% and ventilation 2 to 3%) with *ad libitum* access to feed and water. Lighting was provided for 16 h light (incandescent lighting, 10 lx) and 8 h darkness cycle. Laying hens individually weighed were randomly divided into three experimental groups (C, T1 and T2). For the elaboration of the diet feed formulations used in this experiment, we considered the objective of the experiment, the species, the hybrid, the age and the nutritional requirements of the Tetra SL laying hens (Tetra-SL LL commercial Layer Management Guide, 2007). The layer diets were isonitrogenous and isocaloric and formulated according to recommendations for dietary need of laying hens for various nutrients. Experimental diets were prepared every two weeks to avoid oxidation. Control group (C) was fed a basal diet; while the other two groups were fed diets rich in polyunsaturated fatty acids and antioxidants as follow: 9% rapeseed meal and 3% grapeseed meal (T1) and a diet with 9% flaxseed meal and 3% buckthorn meal (T2). Each hen was fed daily 120 g of a basal diet C, T1 or T2, through individual feed troughs once daily at 08:30 and water was administered using automatic feeders. The basic structure of diets was the same for all three experimental groups,

characterized by 2750 kcal/kg metabolizable energy and 16.50% crude protein.

Egg sampling. A total of 108 eggs were collected after 6-weeks experimental period when the hens were 56 weeks old to evaluate the shelf life on external and internal quality parameters influenced by the storage time and temperature. The fresh eggs for the initial determinations (day 0) were collected from each treatment (n = 6) and measured within 2 h of being laid. The effect of diets on storage time and temperature was determined from a total of 24 eggs/group divided in four batches of 6 eggs. The samples of the 6 eggs from each group were labelled according to date of production and stored in unchangeable conditions, in chambers in a refrigerator (5°C) and at room temperature (21°C) for 14 and 28 days. Relative humidity was regulated at 50 to 60% for all samples.

Evaluation of egg quality. Characteristics were evaluated in individual eggs for external and internal quality traits. The external characteristics of eggs were egg weight (g), shell weight (g), shell strength (kgF) and shell thickness (μm), whereas internal quality parameters include albumen weight (g), yolk weight (g), albumen pH, yolk pH and Haugh unit. The albumen ratio, yolk ratio and shell ratio respectively were calculated with appropriate formulas (Lokaewmanee & Meesri, 2015).

$$Albumen\ ratio = \frac{albumen\ weight}{egg\ weight} \times 100$$

$$Yolk\ ratio = \frac{yolk\ weight}{egg\ weight} \times 100$$

$$Shell\ ratio = \frac{shell\ weight}{egg\ weight} \times 100$$

Egg weight was measured by weighing egg individually using sensitive balance. All egg parameters were measured automatically by an egg multi-tester Egg Analyzer TM, type 05-UM-001, manufactured by Orka Technology Ltd.

Statistical Analysis. The results were statistically analysed to determine the quality traits of eggs over time. All the numerical results obtained were subjected to Matlab & Simulink libraries. The model included the main effects of the storage times and temperatures and the two-way interactions between these factors. To investigate the effects of storage time and temperature on egg quality parameters among the eggs separated into three storage times and two storage temperatures, ANOVA software was used. To this end we use the linear regression model and we obtain the slope (a) and intercept (b) estimates of the model. We also compute the determination coefficient (R^2) in order to show how the variability of our parameters is explained by the linear regression model. Coefficient of determination (R^2) indicates the proportionate amount of variation in the response variable y explained by the independent variables x in the linear regression model. The larger the R^2 is the more variability is explained by the linear regression model. The level of significance was selected at $p<0.05$.

RESULTS AND DISCUSSIONS

The results regarding the effect of storage time and temperature on quality traits of eggs are shown below. With several exceptions, both storage time and temperature significantly ($P<0.05$) affected almost all parameters of internal egg quality. Albumen weight linearly decreased from 0 to 14 days stored at 5°C in all groups, after which the weight was maintained. Significant differences ($P<0.05$) were registered among 0 vs. 14 and 28 days respectively, in all groups (Table 1). Yolk weight, from samples stored at 5°C for first 14 days, registered significant ($P<0.05$) differences only in T1 samples, after that the weight was maintained constant until 28 days. Dramatic deterioration was observed in both albumen and yolk ratio due to the storage time of 28 days at 5°C. The highest albumen ratio was obtained in T1 eggs (59.29%) followed by T2 (57.46%) and C (56.52). In contrast, to the albumen ratio, the T1 yolk ratio, registered the lowest value (22.48%) compared with C (24.38%) and T2 (24.43%). After 28 days of storage at 5°C, albumen weight from C group was significantly ($P<0.05$) lower compared with T1 samples. Similarly, Samli et al. (2005) reported that albumen ratio decreases with 5.40% after a storage period of 35 days at room temperature (15-18°C), in table eggs from 50-weeks old laying hen. Contrary, Panaite et al.

(2020) reported significant increase of yolk weight at 0 days, from eggs enriched, laid by hens fed rapeseed meal, compared with that fed flaxseed meal. Further, albumen weight from eggs stored at 21°C for 28 days, significantly (P<0.0113) decreased in C samples after 14 and 28 days, while those from T1 significantly (P<0.0066) decreased only after 28 days (Table 2). The albumen weight from T2 samples, were not affected by time or temperature. As it was expected, albumen ratio was affected significantly (P<0.05) in C samples (57.63%) compared with T1 (58.57%) and T2 (58.81%) egg, respectively. Albumen weight at 14 and 28 days of storage at 21°C was significantly (P<0.05) lower compared with both T1 and T2 groups. This positive effect could be justified by the antioxidant effect of grapeseed and sea buckthorn meals added in the hens feed, which delayed the protein deterioration.

Table 1. Effects of storage time and temperature on albumen and yolk weight stored at 5°C and regression analysis coefficients (a), (b) and (R^2)

Item	Albumen weight				Yolk weight			
			5°C					
	C	T1	T2	p	C	T1	T2	p
0 days	39.27^{a}	39.79^{a}	37.37^{a}	ns	16.38	15.89^{a}	16.51	ns
14 days	35.17^{b}	35.46^{b}	35.16^{b}	ns	15.63	14.71^{b}	15.79	ns
28 days	34.01^{bA}	35.04^{bB}	34.92^{b}	*	15.00	14.04^{b}	15.31	ns
ratio	56.51	59.37	57.47	ns	24.40	22.54	24.44	ns
SEM	0.641	0.593	0.433	-	0.350	0.277	0.312	-
p	<0.001	<0.0001	0.0146	-	ns	0.0036	Ns	-
			Time					
(a)	38.93	39.81	37.37	-	16.71	15.93	16.79	-
(b)	-0.16	-0.09	-0.09	-	0.04	0.04	0.04	-
R^2	0.87	0.99	0.99	-	0.59	0.93	0.99	-
			Temperature					
(a)	38.11	39.4	37.11	-	16.99	16.11	16.88	-
(b)	-0.16	-0.11	-0.11	-	0.03	0.04	0.04	-
R^2	0.64	0.87	0.99	-	0.78	0.73	0.87	-

$^{a,\ b}$Different lowercase letters indicate significant differences among the means in each column on different storage temperature. ^{AB} Different uppercase letters indicate significant differences among the means in each row between groups; *significant at P<0.05; ns - not significant. C - control diet; T1- diet supplemented with rapeseed and grapeseed meal mixture; T2- diet supplemented with flaxseed and sea buckthorn meal mixture; SEM – standard error of the mean.

Table 2. Effects of storage time and temperature on albumen and yolk weight stored at 21°C and regression analysis coefficients (a), (b) and (R^2)

Item	Albumen weight				Yolk weight			
			21°C					
	C	T1	T2	p	C	T1	T2	p
0 days	39.27^{a}	39.79^{a}	39.46	ns	16.38	15.89^{a}	16.51	ns
14 days	35.08^{bA}	38.35^{B}	38.44^{B}	*	15.32	14.34^{b}	15.47	ns
28 days	35.02^{bA}	37.25^{bB}	37.79^{B}	*	15.11	14.13^{b}	15.21	ns
ratio	57.62	58.56	58.82	ns	25.47	23.47	25.78	ns
SEM	0.645	0.399	0.437	-	0.356	0.0272	0.433	-
p	0.0113	0.0066	ns	-	ns	ns	ns	-
			Time					
(a)	38.92	39.26	39.10	-	16.55	16.39	16.62	-
(b)	-0.19	-0.17	-0.17	-	-0.03	-0.04	-0.03	-
R^2	0.89	0.80	0.99	-	0.89	0.99	0.99	-
			Temperature					-
(a)	37.94	38.4	36.61	-	16.62	16.62	16.42	-
(b)	-0.21	-0.17	-0.17	-	-0.04	-0.04	-0.04	-
R^2	0.68	0.67	0.99	-	0.99	0.99	0.98	-

$^{a,\ b}$Different lowercase letters indicate significant differences among the means in each column on different storage temperature. ^{AB}Different uppercase letters indicate significant differences among the means in each row between groups; *significant at P<0.05; ns - not significant. C - control diet; T1- diet supplemented with rapeseed and grapeseed meal mixture; T2- diet supplemented with flaxseed and sea buckthorn meal mixture; SEM - standard error of the mean.

Yolks weight significantly (P<0.0272) decreased only in T1 samples, as in the case of those stored at 5°C. In terms of yolk ratio T1 eggs (23.46%) maintained the lowest value compared with those from C (25.41%) and T2 (25.79%), which had close average values (Table 2). There were no significant (P>0.05) differences among the groups in terms of albumen or yolk weight. Previously, Lokaewmanee & Meesri (2015) reported drastically (P<0.0001) decreased of albumen (65.53% to 46.40%) ratio and significantly increased (P<0.05) yolk ratio (27.37% to 34.99%) in lutein enriched eggs after only 21 storage days at 30°C. These changes in egg quality traits as albumen and yolk ratio have been reported also by others (Tabidi, 2011; Samli et al., 2005) and were attributed to water loss by evaporation through the pores in the shell and the escape of CO_2 from albumen.

Shell quality, same as weight, was not affected by temperature (5°C) or by storage time. But there were observed some variations in terms of regression coefficients (Table 3). Shell thickness was also not affected, were noted some slightly lower values for all samples. In terms of shell ratio T2 registered the highest value (14.01%) versus T1 (13.61%) and C (13.03%) samples. In contrast, shell strength, increased under the influence of time. The T2 samples had the highest values after 28 days storage time (4.31) compared with C (3.89) and T1 (3.98), but the differences were not statistically (P>0.05).

Table 3. Effect of storage time and temperature on shell weight, thickness and strength stored at 5°C and regression analysis coefficients (a), (b) and (R^2)

Item	Shell weight			Shell thickness			Shell strength		
	5°C								
	C	T1	T2	C	T1	T2	C	T1	T2
0 days	8.83	8.84	8.96	0.35	0.35	0.35	3.63	3.63	3.67
14 days	8.35	8.83	8.84	0.34	0.34	0.34	3.87	3.95	3.97
28 days	8.29	8.33	8.74	0.34	0.32	0.33	3.89	3.98	4.31
SEM	0.127	0.180	0.248	0.005	0.006	0.005	0.242	0.148	0.151
p	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Time								
(a)	9.03	8.81	9.83	0.34	0.32	0.35	3.72	3.58	3.82
(b)	-0.03	-0.01	-0.01	0	0	0	0.01	0.01	0.01
R^2	0.67	0.87	0.99	0.57	0.59	0.69	0.57	0.54	0.59
	Temperature								
(a)	8.97	8.76	9.58	0.35	0.34	0.33	3.77	3.59	3.82
(b)	-0.04	-0.01	-0.01	0	0	0	0.01	0.01	0.01
R^2	0.89	0.64	0.99	0.99	0.99	0.99	0.69	0.77	0.99

ns - not significant. C- control diet; T1- diet supplemented with rapeseed and grapeseed meal mixture; T2- diet supplemented with flaxseed and sea buckthorn meal mixture; SEM - standard error of the mean.

Shell weight from samples analysed under the influence of temperature (21°C) and time (28 days) from C group, significantly (P = 0.0216) decreased after 14 days of storage, while those from T1 and T2, were not affected (Table 4). Shell thickness maintained close values between all groups at all storage times. Similarly, with samples analysed at 5°C for 28 days, shell strength of samples under the influence of temperature (21°C) significantly (P = 0.0130) increased in T1 samples at 28 days compared with those from 0 days. Also, T2 and C samples registered higher values for shell strength after 28 days of storage, but without significance between them. In line with our results, (Lokaewmanee & Meesri, 2015) reported that egg shell quality was not influenced by lutein enriched diet under the effect of storage time and temperature. Silversides & Scott (2001) reported that the weight of the shell increased with age of the hen until 45-weeks, but when considered as percentage of the egg, the shell decreased with increasing age of the hen. Also, Olteanu et al. (2017), reported increased eggshell weight at the end of the trial, when diets rich in PUFA and antioxidants were given to laying hens. As in our study, the changes in egg shell traits are unclear. Contrary to these findings, Samli et al. (2005), reported that shell weight, and other egg parameters significantly (P<0.001) decreased with increased storage time and

temperature in commercial eggs from old laying hens. In the literature is a lack of reports on internal and external quality characteristics of eggs from hens fed diets rich in polyunsaturated fatty acids and antioxidants, in different storage conditions.

Table 4. Effect of storage time and temperature on shell weight, thickness and strength stored at 21°C and regression analysis coefficients (a), (b) and (R^2)

Item	Shell weight (g)			Shell thickness			Shell strength		
				21°C					
	C	T1	T2	C	T1	T2	C	T1	T2
0 days	8.83	8.84	8.96	0.35	0.35	0.35	3.63	3.63[a]	3.67
14 days	8.24[a]	8.55	8.73	0.34	0.35	0.35	3.98	3.99	3.92
28 days	8.07[b]	8.21	8.56	0.33	0.34	0.34	4.10	4.41[b]	4.32
SEM	0.160	0.148	0.204	0.006	0.005	0.006	0.180	0.140	0.165
p	0.0216	ns	ns	Ns	ns	ns	ns	0.0130	ns
				Time					
(a)	8.65	8.79	8.89	0.35	0.35	0.35	3.63	3.64	3.64
(b)	-0.01	-0.01	-0.01	0.00	0.00	-0.01	0.02	0.02	0.02
R^2	0.99	0.98	0.99	0.92	0.93	0.99	0.97	0.99	0.99
				Temperature					
(a)	8.66	8.75	8.75	0.35	0.35	0.35	3.72	3.72	3.71
(b)	-0.03	-0.01	-0.01	0.00	-0.01	-0.01	0.03	0.02	0.02
R^2	0.86	0.86	0.99	0.71	0.99	0.99	0.87	0.89	0.99

[ab]Different letters indicate significant differences among the means in each column on different storage temperature. ns- not significant. C- control diet; T1- diet supplemented with rapeseed and grapeseed meal mixture; T2- diet supplemented with flaxseed and sea buckthorn meal mixture; SEM – standard error of the mean.

As storage time and temperature increased, egg weight significantly (P<0.001) decreased in all groups under 21°C, within 28 days storage time, compared with those stored at refrigerator (5°C). After 14 days at refrigerator, egg samples from C group had a significantly (P<0.05) lower weight compared with those from T1. At the end of storage time (28 days) at 5°C, both T1 and T2 egg weight were significantly higher compared with C eggs. The interaction effects between storage time and temperature were significant for egg weight kept at 21°C, but the interaction effects on egg weight was not significantly decreased by storage from 0 to 14 days at 5°C, significant interaction was observed after 14 days of storage. When the storage temperature was increased to 21°C, however, the egg weight dramatically decreased from 64.09 to 59.02 in C group, from 64.18 to 59.83 in T1 and from 64.17 to 59.38 in T2 (Figure 1A). These results are in agreement with those of Jin et al. (2011) and Samli et al. (2005), who reported significant egg weight reductions of approximately 3% within 10 days of storage at 29°C. Similar weight losses were also reported by Akyurek and Okur (2009). The storage period and temperature significantly affected all freshness parameters of internal egg quality given by HU, albumen and yolk pH (P<0.001). However, the albumen and yolk pH significantly increased with storage time of 28 days and temperature at 21°C. The interaction effects between storage time and temperature were significant for HU, yolk pH and albumen. Significant deteriorations were observed in HU due to storage time of 14 days and temperature of 21°C (Figure 1B). However, the eggs stored at refrigerator (5°C) tended to have higher HU compared with those from initial day. Similarly, previously was reported that by supplementing laying hens' diets with flaxseed-rosehip mixture, or flaxseed-grapeseed mixture, HU increases due to the effect of antioxidants added to delay the yolk lipid oxidation and protein denaturation from albumen (Sărăcilă et al., 2017). Storage at temperatures higher than 5°C for more than 14 days caused considerable deterioration in HU. In all treatments HU decreased significantly (P<0.05) from 86.28 (0 days) to 72.67 (14 days) to 32.12 (28 days) in C group, from 86.70 (0 days) to 75.27 (14 days) to 43.68 (28 days) in T1 eggs, while those from T2 decreased from 86.54 to 75.15 and at day 28 to 44.22 when preserved at 21°C. With respect to the effect of storage time and temperature on the physiochemical properties of eggs, we observed a significant (P<0.05)

increase in albumen pH with increasing storage time and temperature (Figure 1C). The albumen pH was not affected by storage time at 5°C. At 14 days, albumen pH was higher in eggs stored at 21°C, compared with those stored at 5°C. After 28 days, albumen alkalizing was accelerated by increasing storage temperature (21°C) with the interaction of storage period (28 days), registering significantly higher values compared with those stored at 5°C. There were no differences ($P>0.05$) among the groups stored in different conditions. Generally, the increase in albumen pH occurs due to the dissociation of carbonic acid (H_2CO_3), forming water and carbon dioxide (Figueiredo et al., 2013). The pH determination of the albumen is a suitable measure to evaluate the freshness of the eggs, since there is less influence of the strain and age of the bird on the pH compared with other quality measurements (Silversides and Scott, 2001). The albumen pH of the freshly laid egg usually ranges from 7.6 to 8. However, the albumen pH increases with the storage period of the egg, reaching 9.5 while under the high storage temperature the pH can reach over 10 (Alleoni & Antunes, 2001), after that the albumen alkalinization starts to occur due to protein degradation.

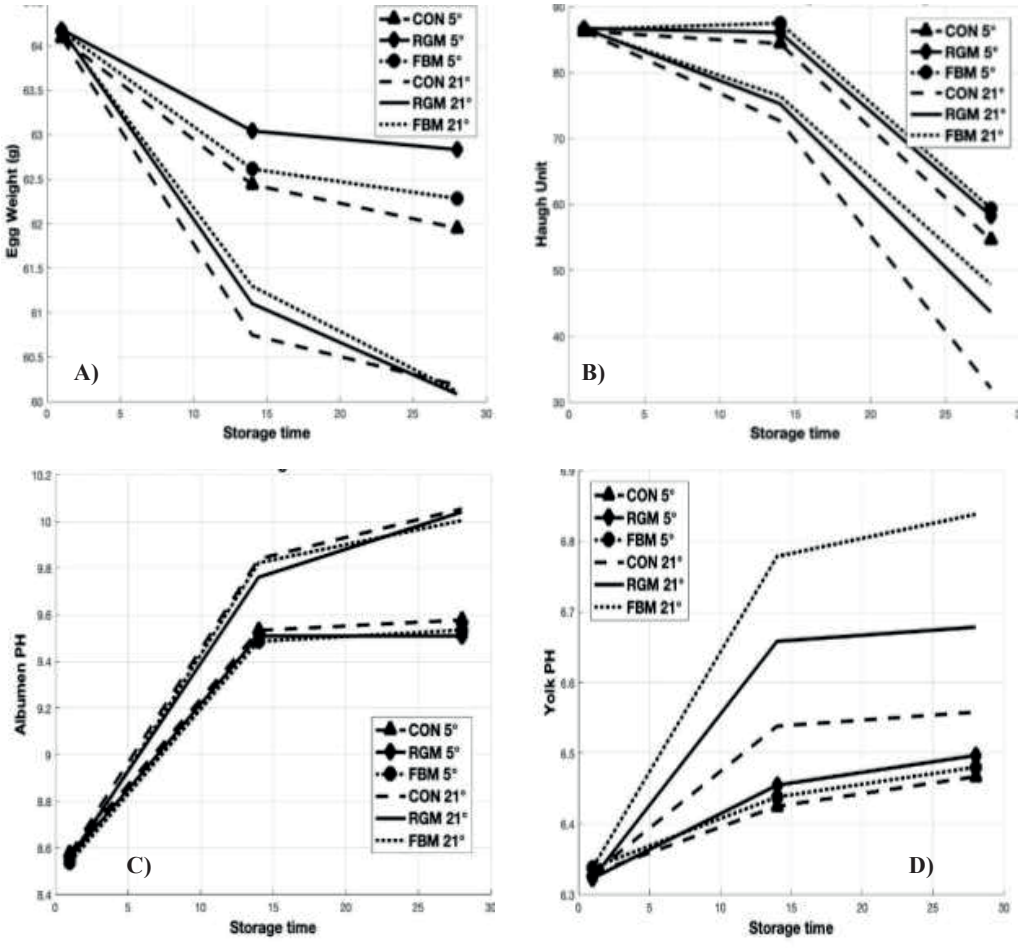

Figure 1. Relationship between storage time (0, 14 and 28 days) and temperature (5°C and 21°C) on freshness parameters: A) egg weight(g); B) Haugh Units (HU); C) albumen pH; D) yolk pH

The yolk pH value as albumen pH increased with increasing storage time (Figure 1D). The yolk pH values from eggs stored at 5°C, increased linearly from 0 to 28 days refrigeration, without significant differences among the groups. On the other hand, eggs stored at 21°C significantly ($P<0.05$) differed among the groups after both 14 and 28 days of storage. Responsible for differences in both T1 and T2 yolk samples at 28 days of storage time, could be the high fatty acid content in the added meals from rapeseed (40.26 to 43.19%) and grapeseed meal (64.71 to 66.60%) in T1 and flaxseed meal (70.23 to 78.80%) and sea buckthorn meal (27.33 to 30.44%) in T2 (Panaite et al., 2016; Vlaicu et al., 2017; Cornescu et al. 2018). It was reported that the increase in yolk pH (usually 6.0) has little variation (6.35 to 6.85) even after long storage periods (Oliveira and Oliveira, 2013). It has been reported that during storage, CO_2 escapes through the eggshell pores. So, the increase in albumen pH over time may be also due to the loss of CO_2 and/or a change in the bicarbonate buffer system (Biladeau and Keener, 2009). Although the pH of the egg albumen and yolk increased along with the storage time and temperature, the changes of albumen pH were not as large as those of the yolk pH. Previous studies reported similar effects on yolk pH, which was significantly affected by storage time (Samli et al., 2005; Akyurel and Okur, 2009; Jin et al., 2011). Increasing storage time and temperature diluted the egg albumen resulting in breakdown of the protein structures of the albumen and vitelline membrane (Jones, 2007) which accelerates to the passage of some components of the albumen pass through the yolk membrane, reducing egg weight and viscosity (Ahn et al., 1999). In this study, storage time up to 28 days and temperature (21°C) significantly ($P<0.05$) affected almost all of the internal and external egg quality parameters. As shown in the presented tables, we calculated the correlation coefficients of storage time and temperature on egg quality traits. Storage time and temperature were negatively correlated with albumen, yolk and shell weight but were positively correlated with egg weight. Among these coefficients, high temperature showed the highest negative correlation with albumen weight, shell weight and thickness in C and T1 after 28 days storage time, which means that temperature, was a more sensitive determinant of egg quality deterioration than storage time. Moreover, temperature was an absolute factor in determining the internal egg quality because the HU dramatically decreased after being stored for 14 to 28 days at storage temperatures up to 21°C. Similar interaction effects also occurred for the albumen pH and yolk pH with increasing storage time and temperature.

CONCLUSIONS

The quality characteristics of eggs were not adversely affected when eggs were stored in refrigerators for 14 days, but room temperature significantly affect some egg quality characteristics by increasing weight loss, yolk weight, yolk pH, albumen pH and by reducing HU during storage for different time intervals. It can be concluded that egg from hens fed diets rich in polyunsaturated fatty acids and antioxidants, should be kept in refrigerators up to 28 days and at room temperature up to 14 days, after that the alteration in albumen and yolk starts to occur, but still maintain relatively good internal quality characteristics for human consumption up to 28 days.

ACKNOWLEDGEMENTS

This work was supported by a grant of the Romanian National of Research and Innovation, CCCDI – UEFISCDI, project number PN-III-P1-1.2-PCCDI-2017-0473, within PNCDI III - PC3.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

RESULTS OF STUDIES ON JUSTIFICATION OF A DEVICE FOR PRODUCTION OF ECOLOGICALLY PURE CREAM

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Abstract

A device for the production of environmentally friendly cream is presented. A research methodology for substantiating optimal parameters is presented. The regression equation describing the productivity of the milk separation process was determined on the basis of the theory of probability and mathematical statistics. When solving it, the optimal design and kinematic parameters of the device for the production of environmentally friendly cream were identified. Its optimal constructive, kinematic and technological parameters were established, which were: the angle formed by the vector of the relative speed and the reverse direction of the vector of the portable speed for the end of the blades βl2 = - 1 deg; drum angular velocity ω = 1130 s^{-1}; milk temperature t = 44ºC. The use of a scraper-cream separator will increase the separation productivity by 14% compared to the serially produced ESB-0.2.

Key words*: cream, cream separator, drum, milk.*

INTRODUCTION

The main product of dairy cattle breeding of agricultural enterprises is whole milk, which, like its components (cream and skim milk), are raw materials for the production of various dairy products. For this reason, cream separators are used as part of most technological lines for the production of dairy products (Melken, 1991). Analysis of their designs allows us to consider one of the main disadvantages of uneven filling of interplate spaces with milk, which is the main reason for the decrease in their productivity. Therefore, the development of new designs of separators, cream separators, contributing to an increase in their productivity is relevant and is of great national economic importance (Yashin, 2014; Yashin, 2015).

MATERIALS AND METHODS

Milk fat is a valuable component of milk. Its mass fraction is about 30% of milk solids. The efficiency of separation depends on seasonal changes in the composition of milk, which directly depend on the nutritional value of feed, lactation period, etc. (Yashin, 2018-2020).

The efficiency of separation is directly influenced by the technological processes used in milk production, and such technological factors as: separation temperature, milk acidity, pollution milk by mechanical impurities, size and density of fat globules, mass fraction of fat in milk, pretreatment, density and viscosity of milk.

In addition, among the main design factors, one can single out: the number and size of inter-plate spaces, the shape of the plate, the direction of milk supply to the drum, the method of feeding milk into the stack of plates, the type of feeding device, etc. The angular velocity of the drum belongs to the kinematic parameter.

The optimum temperature of milk during separation is considered to be from 35 to 45°C. An increase in milk temperature during separation above the specified range contributes to the denaturation of milk proteins, that is, a decrease in size or complete destruction of fat globules. In this case, part of the small fat globules is carried away into the skim milk, while the mud space of the separator drum is quickly filled with the separator mucus formed. There is also frothing of cream and skim milk, which undoubtedly leads to deterioration in the release of fat. Strong foaming of the cream can negatively affect its further processing, since this leads to the formation of fine oil grains (fat lumps). At the same time, some of the small fat globules that still end up in the cream, for example, during

the production of butter, are converted into buttermilk, which reduces the degree of use of milk fat.

When separating milk at a low temperature, energy is saved, the development of microorganisms is slowed down, the fat globules are less affected, therefore, in its structure, the cream is more stable and less susceptible to spoilage. But a significant disadvantage of separating cold milk is a decrease in the efficiency of separating fat, because the viscosity of chilled milk is higher than that of heated milk to a temperature of 35 to 45°C. Since with an increase in the viscosity of milk, the rate of floating of fat globules decreases and, consequently, the possibility of their release from milk.

The acidity of milk, according to GOST R 52054-2003, must be within the range from 16 to 21ºT. Increased acidity leads to partial coagulation of milk proteins. Protein flakes quickly fill the mud space of the separator drum, which entails the carryover of fat globules into skim milk and its contamination with mechanical impurities. To avoid this, with increased acidity of milk, it is necessary to stop the separator for washing more often or use self-emptying separators. To avoid a decrease in the separation efficiency, it is recommended to separate milk with acidity not higher than 20°T.

A constructive diagram of the cream separator (Savvin, 2014, RF patent, 2013) is proposed, the novelty of which is confirmed by the RF patent for invention No. 2539759.

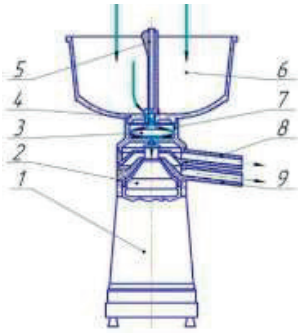

Figure 1. Structural diagram of the cream separator according to the RF patent for invention No. 2539759: 1 - case; 2 - drum, 3 - float; 4 - receiving and output device; 5 - tap; 6 - milk receiver, 7 - float chamber; 8 - cream pipe; 9 - a branch pipe of skim milk

The technical solution is to increase productivity with an admissible sharpness of degreasing, due to the fact that the supply channels of the tray holder of the drum of the separator-cream separator are made expanding towards the periphery and are located along an arc of a circle opposite to the direction of rotation of the drum.

The proposed separator-cream separator (Figure 1) includes a drive located in the housing 1, drum 2, inlet and outlet device 4, consisting of a tap 5 located in a milk receiver 6, a float chamber 7 with a float 3, a cream pipe 8 and a skim milk pipe 9.

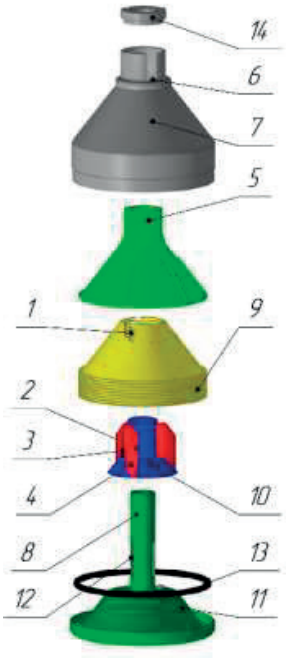

Figure 2. Drum of the separator-cream separator according toRF patent for invention No. 2539759: 1 - slot; 2 - supply channels; 3.4 - blades; 5 - a hole for the withdrawal of cream; 6 - opening for skim milk output; 7 - drum cover; 8 - central tube; 9 - a package of plates; 10 - blade plate holder; 11 - drum bottom; 12 - rectangular hole; 13 - a sealing ring; 14 - nut

The drum of the separator-cream separator (Figure 2) has a cover 7, a central tube 8 with a bottom 11, on which a hollow blade plate holder 10 is placed with a removable package of plates 9, in which rectangular holes 12 are made in the upper part along the height of the package of plates 9, communicating with supply channels 2, formed by blades 3 and 4.

Each plate 9, mounted on a blade plate holder 10, has slots 1 according to the number of supply channels 2 of the blade plate holder 10. Blades 3 and 4 of the plate holder 10 are made expanding towards the periphery and are located opposite to the direction of rotation of the drum along the arc of a circle. In the lid 3 of the drum there are holes 5 and 6 for the output of cream and skim milk, respectively.

RESULTS AND DISCUSSION

The volume of milk to be separated for each experiment was the same and made up the volume of the receiver equal to 5.5 liters.

The separation time was determined as the time from the beginning to the end of the outflow of separation products (skim milk and cream) from the branch pipes.

The milk used in the experimental studies corresponded to GOST R 52054-2003 "Natural cow's milk - raw material". The milk temperature was changed in a water bath. The fat content of the milk was 3.8%. If the requirements for milk in terms of fat content were not met, it was normalized by adding cream with a known fat content or skim milk.

The matrix and the results of the three-factor experiment are presented in Table 1. The results are defined as the average of triplicate

Table 1. Matrix and results of a three-factor experiment

Experience number	Angle between vector the relative velocity and the reverse direction of the vector carrying velocity at the end of the blades	Angular velocity drum	Milk temperature	Performance separator-cream separator m3 / s
	x1	x2	x3	Q
1	1	1	1	0.0000157
2	1	1	-1	0.0000093
3	1	-1	1	0.0000118
4	1	-1	-1	0.0000047
5	-1	1	1	0.0000153
6	-1	1	-1	0.0000093
7	-1	-1	1	0.0000118
8	-1	-1	-1	0.0000047
9	1	0	0	0.0000136
10	-1	0	0	0.0000147
11	0	1	0	0.0000150
12	0	-1	0	0.0000098
13	0	0	1	0.0000148
14	0	0	-1	0.0000108

As a result of the calculations, a two-dimensional section of the response surface was obtained (Figure 3) from the drum angular velocity x^2 and milk temperature x3 at the optimal value angles formed by the vector of the relative velocity and the reverse direction of the vector of the portable velocity for the end of the blades x1.

The obtained values of the factors indicate the finding of an extremum and obtaining the maximum productivity of the separator-cream separator with a paddle plate holder. According to the obtained values, interpolation was carried out for each factor according to Table 1.

Optimal values of factors in the decoded form were: the angle formed by the vector of the relative velocity and the reverse direction of the vector of the portable velocity for the end of the blades $\beta_{л2} = -1$hail; drum angular velocity $\omega = 1130$ from^{-1}; milk temperature $t = 44$ ºC. In this case, the performance of the separator-cream separator is $Q_{с.э.} = 0{,}0000165$ m^3/s.

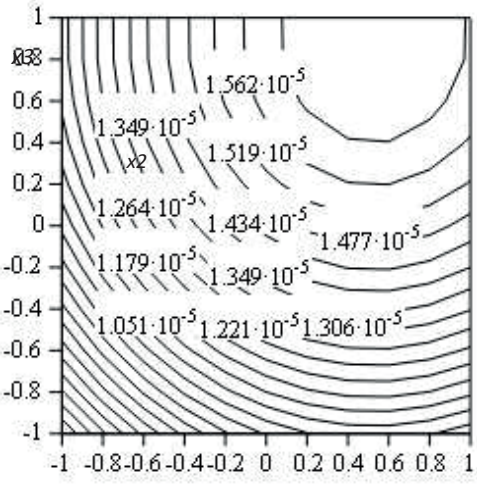

Figure 3. Two-dimensional cross-section of the surface of the response of the performance of the separator-cream separator from the angular speed of the drum x2 and milk temperature x3 at the optimal value of the angles formed by the vector of the relative speed and the reverse direction of the vector of the portable speed for the end of the blades x1

The density of the original milk during the research ranged from 1029 kg/m^3 to 1032 kg/m^3 with a fat content of 3.8%.

The cream obtained during research in industrial conditions (Figure 4) had a fat content of 18 to 41.5%. The severity of degreasing was%, which meets the requirements of GOST 18113-2013 (%).

Figure 4. Cream obtained during research in production conditions

The proportion of fat globules present in skim milk with a radius of $r = 0.5 \cdot 10^{-6}$ m (Figure 5) is at least 97%, which is in good agreement with the calculated value of the critical radius of the fat globule. At the same time, the number of fat globules in cream reaches 150 billion pieces (Figure 6) in 1 ml, and in skim milk 2 billion pcs.

Figure 5. Fat ball in a small square of the grid of the Goryaev chamber when analyzing skim milk

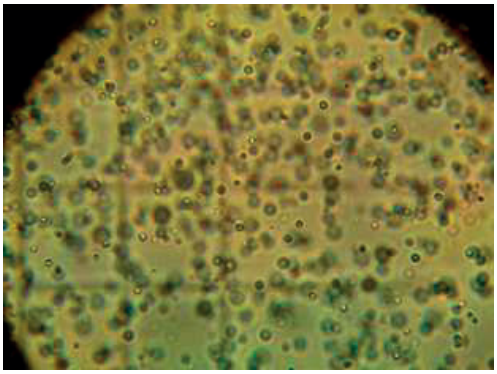

Figure 6. Fat globules in small squares of the grid of the Goryaev chamber when analyzing cream

To determine the mathematical dependence of the performance of the separator-cream separator with a paddle tray on the design, kinematic and technological parameters, and the matrix of experimental studies was decoded with subsequent processing by the Nonlinear Estimation module of the Statistica 6.0 program. The mathematical dependence of the performance of the separator-cream separator

with a paddle plate holder in a decoded form is obtained:

$$Q = -0.0001153 - 0.0000000017 \cdot \beta l2 + 0.00000019 \cdot \omega + 0.00000104 \cdot t - - 0.0000000009 \cdot \beta l22 - 0.0000000001 \cdot \omega 2 - 0.000000012 \cdot t2 ... \quad (1)$$

With optimal design, kinematic and technological parameters, the dependence of the performance of the separator-cream separator with a paddle tray-holder on the number of inter-tray spaces was established when processed by the Non-linear Estimation module of the Statistica 6.0 program. As a result, a mathematical dependence of the performance of the separator-cream separator with a paddle plate-holder on the number of inter-plate spaces was obtained:

$$Q_C = 0{,}000001342 \cdot z \quad (2)$$

The multiple correlation coefficient is R = 0.99, and the F-test = 0.99, which shows the degree of density (spread) of the experimental and calculated values. Consequently, the obtained mathematical dependence (2) adequately describes the results. Thus, the productivity of each individual interplate space is a constant value, which confirms the uniformity of filling the interplate spaces with milk.

The multiple correlation coefficient is R = 0.99 and the F-test = 0.97. Consequently, the resulting model adequately describes the results of the experiments.

The divergence of the performance values of the separator-cream separator with a paddle plate holder obtained from the results of a three-factor experiment with optimal values of design, kinematic and technological parameters $Q_{c.э.} = 0{,}0000165$ m^3/s, as well as according to the theoretical relationship $Q_C = 0{,}0000159$ m^3/does not exceed 4%.

CONCLUSIONS

A prototype of a separator-cream separator with a paddle tray has been developed and manufactured, and the optimal values of the angles formed by the vector of the relative velocity and the reverse direction of the vector of the portable speed for the end of the blades have been determined –1o; drum angular velocity 1130 s^{-one}; milk temperature 44ºC at performance of the separator-cream separator with paddle plate holder 0.0000165 m^3/s. The productivity of each individual interplate space has been established, which is a constant value, which confirms the uniformity of filling the interplate spaces with milk.

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WILD LIFE MANAGEMENT, FISHERY AND AQUACULTURE

Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

THE INFLUENCE OF THE DENSITY OF JUVENILE CARP RAISED IN FLOATING CAGES ON THE CONVERSION EFFICIENCY OF FEED

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Abstract

Fish is an important part of human nutrition, with high biological value, easily digestible, without unfavourable effects to human health. Fish consumption is expected to increase by at least 20% in the next years. Increasing the amount of fish meat can be achieved by intensive aquaculture. The current paper presents a comparative analysis of the results obtained in the intensive growth in floating cages of juvenile carp intended for human consumption at different growth densities. As a result of the experiment, fish that grow at a density of less than 15 kg/m^3 have a lower feed conversion ratio and a higher weekly average growth rate than carp raised at a density higher than 15 kg/m^3.

Key words*: carp, density, feed conversion ratio (FCR), growth, meat.*

INTRODUCTION

Fish aquaculture is the main sector of aquaculture and its objectives are breeding and harvesting of fish species suitable for human consumption (Diaconescu, 2003). The fish breeding takes place in controlled conditions where differentiated technologies are applied specific to each species function of their characteristics (Nicolae et al., 2015). Fish aquaculture can be developed in various conditions like natural ponds or lakes (extensive or semi-intensive aquaculture), in man-made structures (artificial ponds, reservoirs, channels) either extensive or intensive on floating cages Pricope et al., 2012). The floating cages were developed extensively in the last 30 years and now can be find on open seas not only in protected areas near the shore (Cardia & Lovatelli, 2015). Also, a big development happened in the last 20 years with indoor aquaculture for species that have a good market price or for endangered species where breeding programmes were required (Lehmköster, 2013).

The accelerated depletion of wild fish stocks is a consequence of overfishing, lack of sustainable management (fish caught under correct dimensions), climate change, breeding area destruction, high levels of pollution, etc (Jardim et al., 2020; Stavrescu-Bedivan, 2015). Because the above-mentioned factors the specialists are trying to develop new technologies aimed to increase aquaculture production at sustainable costs, with emphasis on food security and quality with regard to animal welfare.

MATERIALS AND METHODS

In the present study, we realised a comparative analysis of results obtained for intensive carp growth in floating cages on Mihailesti Lake at various stocking densities. The aim of our work was to determine the right balance between density, feed consumption and overall weight gain in a determined period.

The fish used in our survey is carp juveniles from the farm with an average weight of 187 grams. The fish were distributed on two cages at the end of September as it follows:

- Cage 1 - 35,515 juvenile fish;
- Cage 2 - 26,438 juvenile fish.

The overwintering period started at the end of October and finished at mild-April. After the overwintering period, a first grading and

counting took place (Figure 1). The fish was redistributed in six cages as it follows:
- Fish from cage 1:
  - Cage 1.1. - 12,625 pcs;
  - Cage 1.2. - 11,100 pcs;
  - Cage 1.3. - 6,798 pcs.
- Fish from cage 2:
  - Cage 2.1. - 9,837 pcs;
  - Cage 2.2. - 9,189 pcs;
  - Cage 2.3. - 5.798 pcs.

Figure 1. Cage division scheme (compared cages are represented by the same colour)

In order to establish the growth dynamic of carp juveniles were fed with the same type of food for 10 weeks until the end of June. After this period, the lots were compared through results analysis. The fish were weight before being distributed on cages and after the redistribution, respecting the actual norms and legislation. The weighting was done with an electronic scale through sampling. For each cage we sampled three times 50 pcs each time. The average weight was establish dividing each weight to fifty and then we accounted for the total of the averages and dived to three. The losses were accounted for by counting and weighing the total of the dead fish during the period. The resulted data was analysed establishing the average, standard deviation, variability coefficient, and the average error. The results significance was tested with the Student test.

RESULTS AND DISCUSSIONS

The feed used in the research period was imported from EU country. For feeding the following factors were taken in account fish weight and water temperature. Function of water temperature the number of feeding sessions can vary from one to five; the optimum period between each meal is directly dependent on water temperature. The minimum period in between meals was there hours (Table 1 and Table 2).

Table 1. Number of meals by temperature

Water temperature	12°C	14°C	16°C	18°C	20°C	22°C	24°C	26°C	28°C	30°C
Number of meals	1	1	1	2	3	4	5	4	3	2
Time of meal administration	11:00	11:00	11:00	10:00	08:00	07:00	07:00	07:00	08:00	10:00
				14:00	13:00	11:00	11:00	11:00	13:00	14:00
					18:00	15:00	14:00	15:00	18:00	
						19:00	17:00	19:00		
							20:00			

Table 2. Recommended feed level, kg feed per 100 kg fish/day

Specification		Water temperature									
Fish weight (g)	Granulation (mm)	12°C	14°C	16°C	18°C	20°C	22°C	24°C	26°C	28°C	30°C
100-300	2	0.75	1.26	2.01	3.02	3.77	4.52	5.03	4.52	4.02	3.02
300-750	4	0.60	1.01	1.61	2.41	3.02	3.62	4.02	3.62	3.22	2.41
750-1500	6	0.48	0.80	1.29	1.93	2.41	2.90	3.22	2.90	2.57	1.93
>1500	8	0.39	0.64	1.03	1.54	1.93	2.32	2.57	2.32	2.06	1.54

The feed should have the following qualities:
- good floatability (fish got accustomed to eating at the surface of the water);
- to have broad spectrum of nutrients as the fish doesn't have any other source of food;
- the packaging should be resistant in order not to break during manipulation and loses to be avoided;
- the storage facility should protect from direct sun and high temperatures.

The feed used has a broad spectrum of components like blood meal, fish meal, rapeseed seed oil, soja extract protein, sunflower extract protein, vitamins, minerals, fibres etc. The gross protein content in feed is 30%, gross fat 7%, fibres 5.5%. The digestible energy is 12.6 MJ for pellets above 2 mm.

After the first distribution Cage 1 was populated with 35.515 pcs of fish with an average weight of 90 grams with a density of 18 kg/ m^3 density while Cage 2 was populated with a number of 26438 pcs of fish with an average weight of 90 grams and a 14 kg/m^3 density. After the overwintering period a 14.05% mortality was registered for Cage 1 and 10.26% for Cage 2 the losses being directly related to stocking densities (Figure 2).

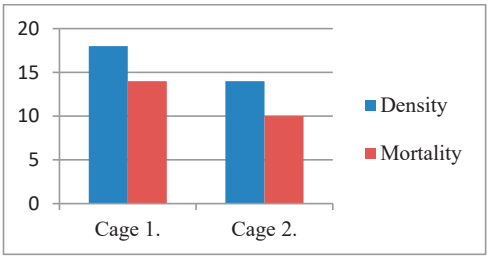

Figure 2. Comparative results on the density and mortality of fish in the two cages

After the overwintering period, the first redistribution of lots took place to be compared after the 10 weeks of feeding.

Cage 1.1. was stocked with 12,625 pcs with an average weight of 120 grams at a density of 7 kg/m^3, Cage 2.1. was stocked with 9,837 pcs with an average weight of 120 grams and a density of 5 kg/m^3. After 10 weeks of feeding Cage 1.1. registered a mortality of 2.3%. The average weight reached 360 g with a feed conversion rate (FCR) of 2.1 using 6,146 kg of feed for a weight gain of 2,927 kg and a final density of 21 kg/ m^3.

Cage 2.1. registered a mortality of 1.7%. The average weight reached 380 grams with a FCR of 1,8 using 4491 kg of feed for a weight gain of 2,495 kg and a final density of 15.8 kg/m^3 (Figure 3).

Cage 1.2 was stocked with 11,100 pcs with an average weight of 200 grams at a density of 8 kg/m^3, Cage 2.2 was stocked with 9,189 pcs with an average weight of 200 grams and at a density of 6.5 kg/m^3.

After 10 weeks of feeding Cage 1.2. registered a mortality of 2.5%. The average weight reached 570 grams with a feed conversion rate (FCR) of 2 using 8,186 kg of feed for a weight gain of 4,093 kg and a final density of 18.5 kg/m^3.

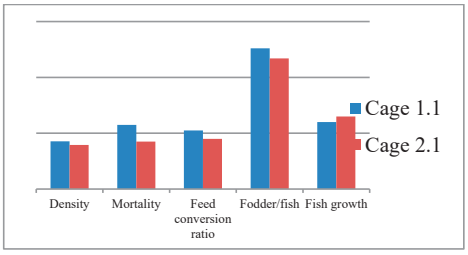

Figure 3. Comparative results between cages 1.1. and 1.2.

Cage 2.2. registered a mortality of 1.8%. The average weight reached 600 grams with a FCR of 1.9 using 6,798 kg of feed for a weight gain of 3,578 kg and a final density of 18 kg/m^3 (Figure 4).

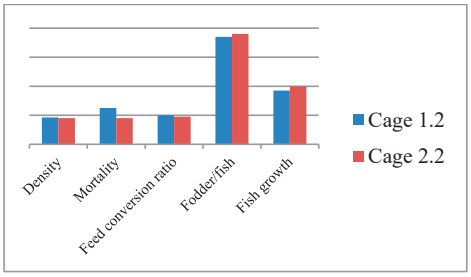

Figure 4. Comparative results between cages 1.2. and 2.2.

Cage 1.3. was stocked with 6,798 pcs with an average weight of 350 grams at a density of 9 kg/m^3, Cage 2.3. was stocked with 5,798 pcs with an average weight of 350 grams and a density of 4.5 kg/m^3.

After 10 weeks of feeding Cage 1.3. registered a mortality of 2.5%. The average weight reached 880 grams with a feed conversion rate (FCR) of 2 using 6,912 kg of feed for a weight gain of 3,456 kg and a final density of 22.5 kg/m^3.

Cage 2.3. registered a mortality of 1.5%. The average weight reached 1.100 grams with a FCR of 1.6 using 6,806 kg of feed for a weight gain of 4,254 kg and a final density of 14.13 kg/m^3 (Figure 5).

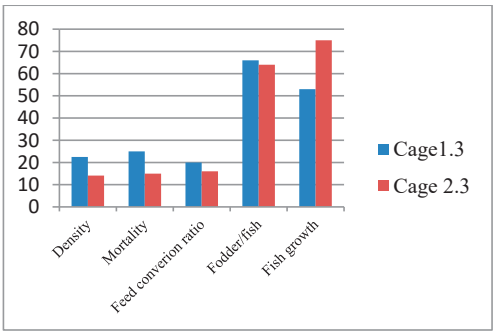

Figure 5. Comparative results between cages 1.3. and 2.3.

The results obtained for carp juveniles growth for a 10 weeks' period on floating cages were evaluated with Student test from relevance perspective.

The lots comparison started from the first stage meaning from the distribution in the 2 cages. The averages for the 2 cages were compared following the three parameters (number of fish, mortality and feed quantity).

As it can be seen in Table 3 for the number of individuals a significant difference was observed but was normal keeping in account that the initial difference was 7,000 pcs.

Table 3. Testing the significance of the results obtained in the formation of the starting cages (1 and 2)

Character		Cage1	Cage 2	t Calculated	t Tabular	Level of significance
Fish number	Average	34455.75	26787.00	17.46	2.57	S
	Variance	536575.58	234996.67			
Mortality	Media	3.85	2.65	0.49	2.57	INS
	Variance	17.80	6.59			

After the feeding period, we analysed the results from cages 1.1. and 2.1. with the Student test and we found significant differences only from number of fish point of view, app 2,500 pcs (Table 4).

Analysing the two cages from mortality point of view we can say that the results obtained are homogenous from averages perspective with a calculated value of 0.68 in comparison with 2.11 the table value. Regarding the weight there are no significant differences, the groups are homogenous although a high difference in number of pcs per cage is registered. As it can be observed in Table 4 the calculated value for the feed quantity was 0.27 compared with 2.1 the table value; it means that from average feed consumption perspective both cages are homogenous.

Analysing the next set of cages 1.2. and 2.2. which were created after the first grading we can observe a consistency in the results with significand variations only from number of fish perspective, app 2,000 pcs (Table 5). The value calculated for the weight of carp juveniles was 1.21 while the table value is 2.12 so we can assess that the two groups are homogenous from weight point of view. The last two indicators analysed are feed quantity and mortality where we didn't register significant variations, meaning that the groups are homogeneous.

Table 4. Testing the significance of the results obtained after the feeding period for cages 1.1. and 2.1.

Character		Cage 1.1	Cage 2.1	t Calculated	t Tabular	Level of significance
Growth	Average	0.207	0.239	0.800	2.120	INS
	Variance	0.006	0.010			
Fish number	Average	12029.500	9550.300	30.230	2.160	S
	Variance	55429.833	11830.455			
Mortality	Average	0.662	0.385	0.686	2.110	INS
	Variance	1.128	0.498			
Feed quantity	Average	500.700	465.400	0.275	2.101	INS
	Variance	77034.456	87990.711			

Table 5. Testing the significance of the results obtained after the feeding period for cages 1.2 and 2.2

Character		Cage 1.2	Cage 2.1	t Calculated	t Tabular	Level of significance
Growth	Average	0.207	0.239	0.800	2.120	INS
	Variance	0.006	0.010			
Fish number	Average	12029.500	9550.300	30.230	2.160	S
	Variance	55429.833	11830.455			
Mortality	Average	0.662	0.385	0.686	2.110	INS
	Variance	1.128	0,.498			
Feed quantity	Average	500.700	465.400	0.275	2.101	INS
	Variance	77034.456	87990.711			

Table 6. Testing the significance of the results obtained after the feeding period for cages 1.3 and 2.3

Character		Cage 1.3	Cage 2.3	t Calculated	t Tabular	Level of Level of significance
Growth	Average	0.484	0.525	0.657	2.110	INS
	Variance	0.015	0.024			
Fish number	Average	6727.700	5539.300	117.502	2.110	S
	Variance	615.344	407.567			
Mortality	Average	0.116	0.117	0.007	2.110	INS
	Variance	0.120	0.078			
Feed quantity	Average	595.800	609.000	0.088	2.101	INS
	Variance	106778.178	115926.889			

As it can be seen in Table 6 there is a significant variation in fish numbers but which is normal as the cages were stocked with app 1000 pcs difference from the beginning of experiment.

Analysing the two cages from mortality point of view we can observe a calculated value of 0.007 in comparison with 2.11 the table value.

This shows that from an average mortality perspective the cages are homogenous. The feed quantity used is similar which again shows homogeneity between the two lots. The average value calculated for carp is 0.65 while the table value is 2.11 which shows homogeneity between the two lots. (Table 6).

CONCLUSIONS

After the research done and the results obtained regarding the technology of carp growth in floating cages the following conclusions emerged:

- the fish density should not exceed 15 kg/m^3 in order to minimize the stress, mortality rates and disease risk;
- the fish grown in densities smaller than 15 kg/m^3 have a bigger growth rate, a better FCR, lower than 2, the economics being significantly improved;
- a lower density generates a more uniform growth between individuals, while a bigger density creates an uneven growth with significant number of fish underdeveloped;
- the fish which are more likely to get diseases are those under one year old.

ACKNOWLEDGEMENTS

This research work was a part from the PhD thesis of Anin Ionut Alexandru - “The influence of thinning and subdivision on carp for consumption increased in floating ponds”, and was carried out with the support of Faculty of Engineering and Management of Animal Production, University of Agronomic Sciences and Veterinary Medicine of Bucharest, Pelicanul Distribution carp farm (Giurgiu County) and Romanian Fish Board.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

THE INFLUENCE OF STOCKING DENSITIES ON GROWTH PERFORMANCE OF COMMON CARP (*CYPRINUS CARPIO*, LINNE 1758) REARED IN A RECIRCULATING AQUACULTURE SYSTEM

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Abstract

To determine the influence of stocking density on the common carp growth performance and body composition, four variants: 0.9 kg m^{-3} (V_1), 1.8 kg m^{-3} (V_2), 2.6 kg m^{-3} (V_3), and 3.5 kg m^{-3} (V_4) was carried out during 31 days. In this context, 2100 common carp fry, with an initial weight of 1.8 g $fish^{-1}$, were maintained in twelve rearing units of a recirculating aquaculture system. Fish were fed three times per day with extruded feed containing 50% crude protein and 14% fat. At the end of the experiment, the fish's growth performance was assessed. Better results were obtained for the V1 variant. Regarding the biochemical composition of fish meat, significant differences ($p<0.05$) were recorded in the water content, while the ash lipids and protein content showed no significant differences ($p>0.05$) between the experimental variants. Therefore, it can be concluded that a stocking density of 0.9 kg m^{-3} is optimal for rearing juveniles of common carp without compromising the specific growth rate (SGR), survival, and biochemical composition of fish meat.

Key words*: biochemical composition, FCR, SGR, stocking density.*

INTRODUCTION

According to FAO, aquaculture is contributing more and more to world production of aquatic food, given that, in the case of the wildest fish stocks, the limits of sustainable exploitation are at present almost touched or even outdated. In 2018, 46% of the total production and 52% from fish for human consumption were assured from aquaculture (FAO, 2020).

In Romania, aquaculture is predominantly freshwater, with the availability of inland waters providing the right conditions for fish farming. According to FAO Fishstat, in 2018, the aquaculture sector from Romania produced 12298 tons.

The most important cultured fish species from our country is common carp (Eurostat, 2017). Usually, it is raised extensively or semi-intensive in polyculture with Asian cyprinids. These growing technologies are based on the natural productivity of the ponds with some additional feed based on local cereals.

Lately, there is also an increasing interest regarding the rearing of carp in intensive conditions, mainly in recirculating aquaculture systems (RAS). Generally, the profitability of these systems depends on the density at which the fish are stocked (North et al., 2006). Therefore, determining the optimum stocking density is the most important criterion for designing an intensive aquaculture system (Summerfelt & Vinci, 2008).

Higher stocking densities can affect digestion and food absorption (Abdel-Tawwab et al., 2014), reduce fish growth performance, survival, size variation, health, and fish mortality (Ruane et al., 2002; Pouey et al., 2011). Also, higher stocking densities lead to deterioration of water quality because of the metabolic excretion of fish (Çağiltay et al., 2017), increase stress (Aksungur et al., 2007), an aspect which can have consequences in the aggressive behavior of fish. According to a study by Firas et al. (2020), at higher stocking densities, fish spent more time feeding and swimming and less time resting, an aspect that can negatively affect fish growth. On the other hand, lower stocking densities may reduce the overall production (Apu et al., 2012), causing economic losses.

Various studies have been carried out about the effects of stocking density on fish growth performance for different species such as

rainbow trout (Sirakov & Ivancheva, 2008; Mocanu et al., 2011; McKenzie et al., 2012), Atlantic salmon (Wang et al., 2019), African catfish (Van de Nieuwegiessen, 2009) and so on. Positive or negative effects on growth performance have been reported from these studies, and the pattern of this relationship appears to be species-specific. That is why, to maximize the production and profitability of a RAS system, it is important to determine the optimum stocking density suitable for each species and each growing stage.

Although carp is a fish raised worldwide, the information about the optimal stocking density practiced in the RAS is limited or non-existent. Therefore, this study aimed to investigate the effects of stocking densities on the growth performance, survival, and biochemical composition of common carp, with an initial weight of 1.8 g, reared in a recirculating aquaculture system.

MATERIALS AND METHODS

Experimental design. The present study was conducted for 31 days in a recirculating aquaculture system (RAS) at the Faculty of Food Science and Engineering, University Dunărea de Jos, Galați, România. The RAS system is provided with twelve rearing units, with a volume of 0,132 m^3 each. The recirculating system was described in detail in the paper of Mocanu et al. (2011).

A total of ţ2100 fishes with an initial weight of 1.8 g $fish^{-1}$ were stocked in the rearing units of the RAS system to create four experimental variants: V_1-70 fish, and the initial stocking density of 0.9 kg m^{-3}, V_2-140 fish, and the initial stocking density of 1.8 kg m^{-3}, V_3-210 fish and the initial stocking density of 2.6 kg m^{-3}, and V_4-280 fish and the initial stocking density of 3.5 kg m^{-3}. The experiment was conducted in triplicate.

Fish were fed three times per day with a diet containing 50% crude protein and 14% fat at a feeding level of 5% BW day^{-1} (Table 1). During the experiment, fish were kept under a natural photoperiod of approximately 12/12 h light/dark cycle.

Water quality parameters such as dissolved oxygen, temperature, and pH were recorded daily with the help of Hannah 98194. Simultaneously, the concentration of nitrogen compounds was measured twice per week with the help of the Spectroquant Nova 400 photometer with Merck kits.

Table 1. Ingredients of the experimental diet

Composition	Quantities
Crude protein	50%
Fat	14%
Crude cellulose	2%
Digestible energy	4100 kcal kg^{-1}
Lysine	2.5%
Phosphor	1%
Copper	6 mg
Vitamin A	20000 UI kg^{-1}
Vitamin D3	2000 UI kg^{-1}
Vitamin E	200 mg kg^{-1}
Vitamin C	200 mg kg^{-1}
Ingredients: fish meal, poultry meal, corn gluten, wheat gluten, wheat flour, animal fat, feed yeast, hemoglobin, vitamins, minerals.	

Fish growth performance. At the end of the experiment, the following technological efficiency indicators were calculated: growth rate, food conversion ratio, specific growth rate, and the protein efficiency ratio using the following equations:

- ✓ Weight Gain (W) = Final Weight (W_t) – Initial Weight (W_0) (g);
- ✓ Food Conversion Ratio (FCR) = Total feed (F)/Total weight gain (W) (gg^{-1});
- ✓ Specific Growth Rate (SGR) (%Body weight day^{-1})) = $[(\mathrm{Ln}W_t - \mathrm{Ln}W_0)/t] \times 100$.

Somatic measurements were made at the end of the trial at 50 fish/experimental variant. Total length (TL) and body weight (BW) for each variant were used to determine the relationship $W=a\times L^b$, where "a" is the intercept (the initial growth coefficient), and "b" is the allometric coefficient (Ricker, 1975). The coefficient of variation (CV, %) was calculated as the ratio of the standard deviation to the mean of weight to have a measure of fish dispersion.

Proximate analysis of fish. To determine the biochemical composition of fish, samples were taken both in the initial and final stages of the experiment. The proximate composition of fish was analysed using the AOAC (2000) method. The chemical composition of meat crude protein was analysed according to the Dumas method (N×6.25), and crude lipids were determined by the Soxhlet method, using petroleum ether as a solvent.

Dry matter was determined by drying the samples at 105 ± 2ºC using Jeio Tech Convection Oven, and ash was evaluated by calcification at temperatures of 550 ± 20°C in a Nabertherm furnace.

The main indicators used for the evaluation of biochemical fish compositions were as follows:

- ✓ Protein efficiency ratio (PER): PER = (Bf-Bi)/(F×PB), where: F = quantity of administrated fed (kg), PB = amount of fed protein (%);
- ✓ Protein utilization efficiency (PUE): PUE = 100×(W×Pf–Wi×Pi)/(F×Pb) (%), where: Pf - muscle tissue protein at the end of the experimental period (%); Pi - muscle tissue protein at the initial stage of the experimental period (%); Wf - final biomass (kg); Wi - initial biomass (kg); F - total feed quantity consumed (kg); Pb - administrated feed protein concentration (%).

Data analysis. Data were analysed using SPSS 21 for Windows. Data regarding fish growth performance and the biochemical composition were expressed by average and standard deviation (Average ± SD). Kolmogorov-Smirnov tests determined the normality of the data used for analysis. One-way ANOVA and Duncan's multiple range tests were used to compare the differences between the experimental groups. Significance was determined at $\alpha = 0.05$.

RESULTS AND DISCUSSIONS

Water quality. It is well known that water quality has a significant impact on the fish's biology and physiology, affecting the health, welfare, and productivity of a fish culture system. Generally, at higher stocking densities, lower growth performance is also determined by an increased production waste production rate.

Water chemical parameters during the experimental period are presented in Table 2. In our recirculating system, all the water parameters were reasonably constant during the experimental period and were not affected by stocking density (ANOVA, $p>0.05$).

Table 2. Synthetic table with the average values (± SD) of the main physicochemical parameters of water

Parameters	V_1	V_2	V_3	V_4
Temperature °C	21.2±0.18	21.6±0.11	21.4±0.12	21.6±014
Dissolved oxygen (mg L^{-1})	7.71±0.12	7.23±0.09	7.10±0.12	7.05±0.08
pH (pH units)	7.62±0.11	7.32±0.09	7.41±0.11	7.29±0.10
N-NO_3^- (mg L^{-1})	21.13±0.20	19.1±0.7	18.5±0.36	17.9±0.65
N-NO_2^- (mg L^{-1})	0.03±0.02	0.04±0.01	0.05±0.02	0.05±0.01
N-NH_4^+ (mg L^{-1})	0.17±0.02	0.22±0.05	0.19±0.03	0.19±0.04
P_2O_5 (mg L^{-1})	5.90±0.19	5.53±0.35	5.2±0.43	5.76±0.32

Note: Data are presented as triplicate mean ± SD.

Water temperature was around 21.2 ± 0.18 °C in V_1, and 21.6 ± 0.14 °C in V_4, dissolved oxygen content varied between 7.05 ± 0.08 mg L^{-1} in V_4 and 7.71 ± 0.12 mg L^{-1} in V_1. Water pH in the recirculation system was kept constant, and the lowest values were recorded in the V_4 variant (7.29 ± 0.10 pH units). Also, the nitrogen compounds were in the optimum interval for the cultivated fish species. The maintenance of these optimum water concentrations was possible by the benefit of optimized technical and technological parameters of the RAS. The fish rearing units were cleaned daily, and only 10% of fresh water was added to the RAS system. Also, a significant role for the maintenance of optimum water chemical parameters in RAS during the experimental period has the mechanical and biological filters which conditioned the water properly.

Fish growth performance. The mean final weight, mean weight gain, percentage survival rates, FCR, and SGR of fish in all the treatments at the final of the experiment are presented in Table 3.

Table 3. Technological performance indicators obtained at the end of the experimental period

Growth performance	V_1	V_2	V_3	V_4
Initial biomass (g)	126	252	378	504
Initial biomass (kg m^{-3})	0.9	1.80	2.60	3.50
The initial number of fish	70	140	210	280
Initial weight (g $fish^{-1}$)	1.80±0.00	1.80±0.00	1.80±0.00	1.80±0.00
Final biomass (g)	313.67±6.66	521±12.77	692±20.52	879.67±28.50
Final biomass (kg m^{-3})	2.20±0.05	3.65±0.09	4.84±0.14	6.16±0.20
The final number of fish	68.00±1.0	122±3	171.67±6.51	221.67±6.03
Final weight (g $fish^{-1}$)	4.61±0.03*	4.27±0.08**	4.03±0.15***	3.97±0.02***
Weight gain (g)	187.67±6.66*	269.00±12.77**	314±20.52***	375.67±28.50****
Weight gain (kg m^{-3})	1.31±0.05*	1.88±0.09**	2.20±0.14***	2.63±0.20****
Individual weight gain (g)	2.81±0.03*	2.47±0.08**	2.23±0.15***	2.17±0.02***
Survival rate (%)	97.14±1.43*	87.14±2.14**	81.75±3.1***	79.17±2.15***
SGR (% day^{-1})	2.94±0.07*	2.34±0.08**	1.95±0.10***	1.80±0.10***
FCR(g g^{-1})	1.84±0.06*	2.57±0.12**	3.31±0.22***	3.69±0.28****

Note: Data are presented as triplicate mean ± SD.

Despite the fact that all the water parameters were adequately maintained in all treatment groups, in the present study, stocking density affects the growth performance of common carp, being observed a negative correlation between stocking density and growth performance.

The data obtained for each experimental variant regarding the final fish weight showed no deviations from the normal distribution ($p>0.05$ with Kolmogorov–Smirnov test) that permitted us to apply the parametric tests further. One-way ANOVA used at the end of the experiment showed significant differences between the final weight of fish (ANOVA, $p<0.05$). So, the mean final weight of fish at the end of the 31-day experimental period was as followed: V_1-4.61±0.03 g, V_2-4.27±0.08 g, V_3-4.03±0.15 g, and V4-3.97±0.02 g. The post hoc Duncan analysis showed that the final weight of fish from V_1 and V_2 was significantly higher than those of fish from V_3 and V_4.

At the end of the experiment, total length (TL)-weight (W) regressions were plotted (Figures 1-4) to obtain more information about the growth patterns of the fish.

The slope (b) values obtained for all the experimental variants showed a negative allometric growth indicating that the fish length was higher than the body mass. However, in this study, the length-weight relationship was found to be highly correlated, and all values of the coefficient of determination were greater than 0.80.

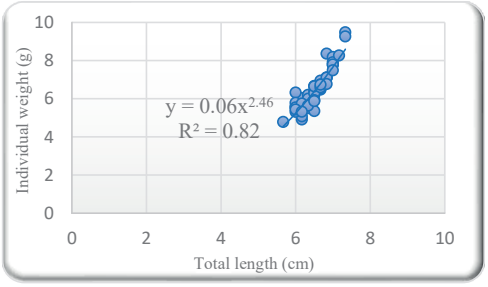

Figure 1. Length-weight regression at the end of the experiment for the V_1 variant (n = 50 fish)

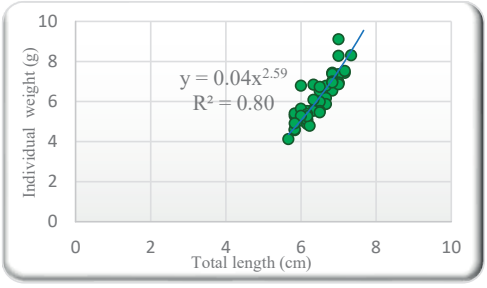

Figure 2. Length-weight regression at the end of the experiment for the V_2 variant (n = 50 fish)

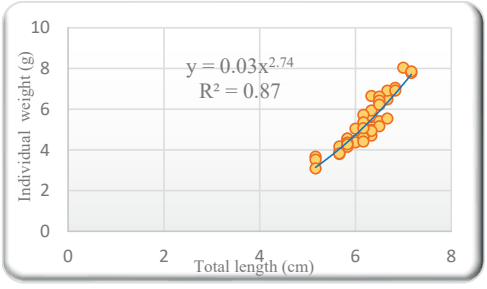

Figure 3. Length-weight regression at the end of the experiment for the V_3 variant (n = 50 fish)

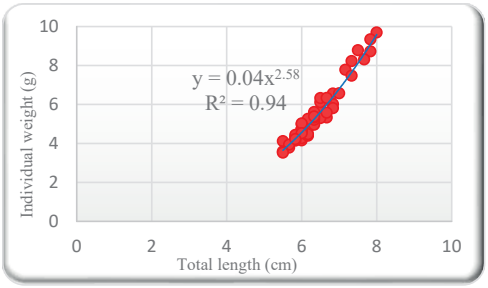

Figure 4. Length-weight regression at the end of the experiment for the V_4 variant (n = 50 fish)

The coefficient of variation (CV) showed higher variability in the V_4 variant (36.35±3.88%), followed by the V_2 (32.66±7.06%) and V_3 (32.53±3.10%). The lowest CV was obtained in V_1 (CV=24.47±3.11%).

Results showed that increasing stocking density results in higher variation in individual growth. An increase of the CV over time indicates inter-individual competition within the fish group (Azaza et al., 2013). Obtaining a higher variability at higher stocking density is undesirable in aquaculture, preferable to reduce fish size variations and obtain homogeneous fish size, which facilitates feeding, harvesting, marketing, and processing (Azaza et al., 2010; Azaza et al., 2013).

The individual weight gain of fish was significantly affected by stocking density (ANOVA, $p<0.05$). Duncan's multiple range tests showed three distinct groups: the individual weight gain of fish from V_1 was significantly different from those of V_2. In contrast, the individual weight gain of fish from $V_3$and V_4 was similar.

Survival is a crucial indicator of fish health status (Rey et al., 2019). In our experiment, it can be observed that the survival rate was directly influenced by the stocking density, and significant differences (ANOVA, $p<0.05$) were obtained. The post hoc analysis showed that the survival rate of fish from the V_1 (97.14±1.43%) group was significantly higher than that from V_2 (87.14±2.14%), while no significant differences ($p>0.05$) were recorded between the V_3 (81.75±3.1%) and V_4 (79.17±2.15%) groups.

Also, in SGR and FCR, the best values were obtained in the lowest stocking density (V_1). The average specific growth rate was significantly higher in V_1 (2.94±0.07% day^{-1}). The post hoc analysis showed that the SGR values from V_1 were higher than V_2 (2.34±0.08% day^{-1}), while in V_3 (1.95±0.10% day^{-1}) and V_4 (1.80±0.10% day^{-1}), the values are similar ($p>0.05$).

Regarding FCR, Duncan's multiple range test divided the obtained values into four distinct groups, the best values being obtained in V_1. The FCR ranged from 1.84±0.06 in V_1 to 3.69±0.28 in V_4 and increased with an increase in fish stocking density. Therefore, the higher FCR values obtained at the highest stocking densities indicate low food utilization efficiency.

In the present study, a negative correlation was observed between the stocking density and fish growth performance. The effect of stocking density on growth is in line with the results obtained for other cultured fish species. HtayHtay et al. (2019) conducted a study over five months stocking carp as follows (with an individual weight of 0.5-1.6 g, and standard length 2.2-4.9 cm): 5 fish per tank, ten fish per tank, and 15 fish per tank (water volume 40 L/tank). After five months, the best results for fish survival and growth performance were observed in the variant with the lowest stocking density (5 fish/tank). Also, Marandi et al. (2018), reported after 45 days of growing, better values of FCR and SGR for common carp (initial weight of 1.41±0.5 g fish^{-1}) at lower stocking densities (20 fish/tank, or 0.70 g L^{-1}).

The proximate composition of common carp reared at different stocking densities is presented in Table 4. Generally, fish's biochemical composition is influenced by many factors that depend especially on species, size, age, environmental conditions, and feeding (Cho, 2001).

The results showed significant differences (ANOVA, $p<0.05$) in fish water content the percentage between the four stocking densities. So, the water content from V_1 and V_2 was significantly different from the water content from V_3 and V_4 variants. A significant increase in water content was observed in V_1, V_3, and V_4 variants compared with the initial moment.

Regarding the protein, lipids, and ash content, no significant differences (ANOVA, $p>0.05$) were recorded between the four stocking

densities, but significant differences were recorded compared to the initial moment (ANOVA, $p<0.05$). Thus, there was a significant increase in protein and lipids' content and a significant decrease in ash content.

Table 4. The proximate composition of common carp meat reared at different stocking densities

Parameters	Experimental variants				
	Initial	V_1	V2	V_3	V_4
Water (%)	76.11±0.18	76.48±0.07^{ac}	76.33±0.22	75.20±0.22	75.32±0.08
Protein (%)	12.29±0.18	13.15±0.02^{bc}	13.26±0.02	13.15±0.16	13.53±0.47
Water/protein	6.19±0.03	5.81±0.05^{bc}	5.75±0.02	5.71±0.05	5.57±0.20
Lipids (%)	7.78±0.07	8.80±0.08^{bc}	8.71±0.2	8.81±0.21	8.63±0.21
Ash (%)	2.19±0.10	1.42±0.02^{bc}	1.39±0.07	1.46±0.03	1.44±0.03

Note: Data are presented as triplicate mean ± SD;
a-significant differences between the experimental variants ($p<0.05$); b-insignificant differences between the experimental variants ($p>0.05$).
c-significant differences from the initial moment ($p<0.05$); d-insignificant differences from the initial moment ($p<0.05$)

The water to protein ratio is a suitable instrument to detect excessive water, being more precise and reliable than the water content itself (Manthey-Karl et al., 2012). If the value of the Water/Protein ratio is lower, the nutritional value is higher. The applied stocking densities on common carp have no significant differences (ANOVA, $p>0.05$) in the water to protein ratio. Compared with the initial moment, the nutritional value of fish meat reflected by the water/protein ratio was significantly better (ANOVA, $p<0.05$). At the beginning of the experiment, the water/ protein ratio was 6.19±0.03, and at the end of the experiment, this ratio decreased for all groups, reaching 5.81±0.05 in the V_1, 5.75±0.02 in V_2, 5.71±0.05 in V_3, and 5.57±0.20 in V_4.

To have more information regarding the fish protein gain, we calculate the protein efficiency ratio (PER) and the productive protein value (PUE) (Figure 5).

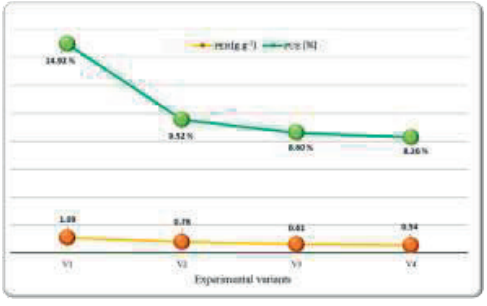

Figure 5. The protein efficiency ratio (PER) and protein utilization efficiency (PUE)

ANOVA test revealed significant differences ($p<0.05$) in PER and PUE values. Duncan's multiple range tests showed that the PER values from V_1 were significantly different ($p<0.05$) from those obtained in V_2, while in V_3 and V_4, no significant differences were obtained in the PER values.

Also, significant differences (ANOVA, $p<0.05$) were obtained between the values of protein utilization efficiency. The evolution of PUE emphasizes a better protein valorisation inversely proportional to the increase of the stocking density. Duncan's multiple range tests revealed four distinct groups belonging to each tested stocking density.

Our values obtained by us regarding carp carcass's biochemical composition are similar to those obtained from other authors. In a study conducted by Khushwinderjit et al. (2018), the biochemical composition of flesh of the common carp fingerlings (with the weight between 5.41-5.49 g, and length between 6.61-6.74 cm) fed with diets replacing protein of plant origin with animal protein in the form of fish silage at different levels, was as follows: water content ranged between 78.20-81.43%, crude protein 13.90- 16.50%, fat 1.60-2.50%, and ash between 1.06-1.60%.

CONCLUSIONS

The effects of stocking density were evident in the growth of common carp in weight gain and the final weight of fish. The best stocking density concerning growth performance and feed conversion efficiency was at 70 fish per rearing unit, with the initial stocking density of 0.9 kg m^{-3}. However, the final results regarding the fish-stocked weight were still low (0.9 kg m^{-3}). This study, therefore, recommends further

research with lower stocking densities, which would result in higher final productions.

ACKNOWLEDGMENTS

The principal author of the article thanks to the "Dunărea de Jos" University of Galați, which through the University Degree Program and the doctoral studies contract has supported the achievement. Also, the authors are grateful for the technical support offered by MoRAS through the Grant POSCCE ID1815, cod SMIS 48745 (www.moras.ugal.ro).

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

THE COMBINED EFFECTS OF STOCKING DENSITY, FEEDING REGIME AND INITIAL SIZE ON GROWTH PERFORMANCE OF RAINBOW TROUT FINGERLINGS

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Abstract

The main purpose of the present experiment is to evaluate if the growth performance, feed utilization and survival rate of rainbow trout (Oncorhynchus mykiss) fingerlings are influenced by factors such as initial stocking size, stocking density, diet or their interaction. This study was based on a 3x3x2 factorial design with three rearing densities (D1 = 2.61±0.22 kg/m^3, D2 = 3.90 ±0.06 kg/m^3 and D3 = 4.80±0.08 kg/m^3), three size classes (SC1 = 2.22±0.24 g/ex, SC2 = 4.90 ±0.18 g/ex and SC3 = 6.87±0.016 g/ex) and two levels of dietary protein (LP = 45%, HP = 50%). After an experimental period of 70 days, rainbow trout growth performance was evaluated through analysis of various technological indicators: IWG (individual weight gain), FCR (food conversion ratio), SGR (specific growth rate), Relative Growth Rate (RGR), PER (protein efficiency ratio). The statistical analysis of data showed significant differences regarding growth among variants both in terms of density and size class. Diet and fish size factors, and the interaction between these, contributed significantly to the variation in protein efficiency ratio.

Key words*: feeding regime, rainbow trout, stress density.*

INTRODUCTION

Rainbow trout has become an important cold-water species for world aquaculture due to accessible rearing technology and its adaptability to different production systems. In Romania, trout aquaculture is practiced mainly in intensive flow-through systems and recirculating aquaculture systems. Despite the high demand for trout on the market, the production within the country is still low (2984.28 tons/year).

The main goal of the trout aquaculture sector is to maximize production efficiency to be competitive on the market in terms of both aspects of fish quality and fish price. Optimizing production depends though on several factors such as fish genetics, feed quality and feeding management, the water quality, size and form of the rearing tanks, stocking density, and size of the fish (Bucur et al., 2017; Luna et al., 2020; Arifin et al., 2019; Lhorente et al., 2019; Kok et al., 2020).

Each of the aspects mentioned above has been studied in recent decades, the aspects related to the nutrition of rainbow trout benefiting the most from the attention of the scientific community (Kiron et al., 1995; Murai, 1992; Ma et al., 2019; Kamalam et al., 2020). Therefore, through in-depth research, quantitative data on nutrient requirements for optimal growth and welfare are already available (NRC, 2011). The amount of feed required depends on water temperature and fish size, smaller fish needing more feed relative to their body due to faster metabolic rates.

Given the growing demand for fish, aquaculture technologies have evolved to intensify. Due to space and environmental constraints that limit the development of new farms, most producers have increased the stocking densities of existing facilities, expecting to maintain growth performance (Fornshell, 2002).

In many cultivated fish species, however, growth is inversely related to stocking density and this is mainly attributed to social interactions or poor water quality as a consequence of metabolites accumulation (Costas et al., 2008; Santos et al., 2010; Tolussi

et al., 2010; Liu et al., 2014; Hosfeld et al., 2009; North et al., 2006).

Increasing densities keeping the same water flow usually reduces fish swimming activity which leads to decreased fitness (both physical and reproductive), growth, survival, and muscle quality (Alsop et al., 1997; Palstra and Planas, 2013).

In practice, different factors have simultaneous effects on the growth performance and, therefore, there are still many aspects of their interaction pending to be elucidated.

In a farming context, a feeding strategy involving the use of specific purpose feeds during a particular production phase is increasingly gaining importance as a tool to influence product quality and environmental compliance (Fornshell, 2002). The expansion and intensification of rainbow trout farming, like for most farmed animals, primarily depends on nutrition and feeding, and continuous research and development are needed to address new challenges to reduce feed costs from current farm levels and increase profitability.

The main objective of the present study was to assess the main and cumulated effect of stocking density and fish size with protein intake on the growth performance of rainbow trout, *Oncorhynchus mykiss*, cultured in a production flow-through aquaculture system.

MATERIALS AND METHODS

Fish and feeding regime

An experiment was conducted in outdoor raceways with flow-through system at the Gilau Fisheries Research Station, to evaluate the effect of stocking density and protein intake on growth and survival of different size groups of rainbow trout fingerlings. The experiments took place in 36 fry rearing basins, in duplicates allowing the installation of multifactorial experiment of 3 x 3 x 2 variables (stocking density x class size x fodder). The basins with a surface of 2 m^2 and a depth between 0.3 and 0.5 m having a total water volume of 0.85 m^3, were populated with 22128 trout fingerlings. The fish were fed with 2 commercial pellets with different protein content, with a daily ratio of 3% of body weight administered 3 times per day (Table 1). The three class sizes, hereby noted with SC were represented by cohorts with mean individual weight of 2.22±0.24 g/ex (SC1), 4.90±0.18 g/ex (SC2) and 6.87±0.016 g/ex (SC3) respectively. Each of the three size classes were subjected to three stocking densities (D) of 2.61±0.22 kg/m^3 (D1), 3.90±0.06 kg/m^3 (D2) and 4.80±0.08 kg/m^3 (D3). The trial was undertaken for 70 days.

Growth performance assessment

In the end of the experiment the fish were weighed and the growth performance of the fish estimated with the following indexes:

Individual Weight Gain (IWG) = Final Weight (W_t)–Initial Weight (W_0) (g)

Food Conversion Ratio (FCR) = Total feed (F)/Total weight gain (W) (g/g)

Relative Growth Rate (RGR) = (W_t–W_0)/t/BW) (g/kg/d)

Specific Growth Rate (SGR) = 100 x (ln W_t–ln W_0)/t (% BW/d)

Protein efficiency ratio (PER) = Total weight gain (W)/amount of protein fed (g)

Relative Weight Gain (RWG%) = (W_t–W_0) x 100/Wt

Table 1. Biochemical composition of fodder pellets

Chemical composition	LP	HP
Protein	45%	50%
Fat	20 %	20 %
Cellulose	17.9%	0.7%
Ash	7.1 %	9 %
Phosphorus	1.0%	1.3%
Vitamin A	10000 UI/kg	12000 UI/kg
Vitamin D3	2000 UI/kg	1800 UI/kg
Vitamin E	200 mg/kg	180 mg/kg
Vitamin C	280 mg/kg	500 mg/kg
Coper sulfate	10 mg/kg	8,5 mg/kg

Statistical analysis

The data normality was confirmed by Shapiro-Wilk test. All data were analysed with 2-Way ANOVA (size and stocking density as the independent variables). If there was an interaction effect of the independent variables on the measured parameters, the data were subjected to one-Way ANOVA. Duncan's multiple range test was used as a post hoc test to compare between means at $P < 0.05$. Data are presented as mean ± S.D. Data analyses were conducted with the SPSS (version 13.0) software.

RESULTS AND DISCUSSIONS

Growth performance of different size rainbow trout, O. mykiss fingerlings, held in different stocking densities and fed diets containing different levels of dietary protein over the 10-week feeding trial is presented in Figures 1 and 2 and Table 2.

Averaged over all treatments, the survival rate was 92.9% being slightly lower, unsignificant ($p<0.05$), when groups from different density variants of the same size group were compared. The highest mortality, however, was correlated with the lowest initial size, for both diet variants, with the highest value for the smallest group fed a low protein diet (Figure 1).

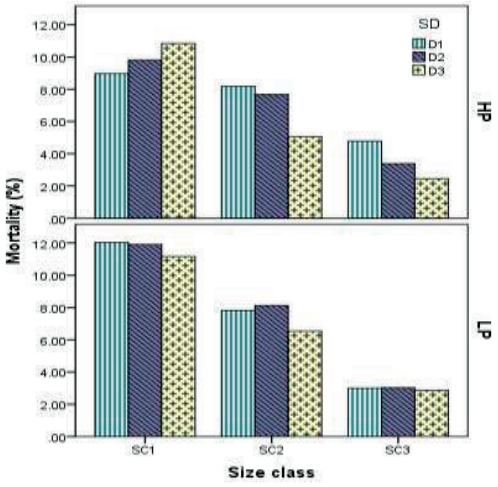

Figure 1. Mean mortality (%) over the experimental trial in all groups

For medium and large-size fish, fed with a high protein diet, slightly higher mortality for the lower densities was observed. This situation could presumably due to the formation of social hierarchies in less crowded fish (North et al., 2006), salmonids having a higher tendency comparing with other species for social dominance (Castanheira et al., 2017), or higher residual nitrogen accumulation and water quality depreciation (Person-Le Ruyet et al., 2008).

In terms of mortalities, the values reached 10.78 % for the smaller fish groups comparing with only 7.23% and 3.25% for the middle size and larger fish. However, for the present study, there was no evidence that higher density induced mortality.

The final mean individual weight did not differ significantly (Anova, $P>0.05$) among density groups but did varied (Anova, $P>0.05$) among size classes (Figure 2).

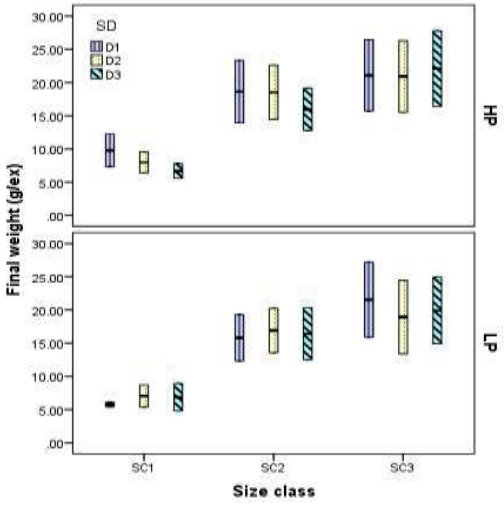

Figure 2. Final individual weight of the trout fingerlings reared in different experimental conditions

At low stocking, densities fish were affected positively in a significant increase in growth in special for smaller fish (Table 2). Thus, mean IWG values over density treatment registered by the SC1 fish fed with high protein were 31 to 35% higher comparing with mean values registered for the fish fed with a low protein diet. Interestingly, for larger fish variants (SC2 and SC3) there were no statistical differences (Duncan test, $P>0.05$) between groups from the same density irrespective of received feed.

Feed is the main input for fish production, feed cost representing almost half of the variable costs during the production cycle (Engle et al., 2020). Feed conversion ratio- FCR is a measure of the feeding efficiency and therefore a lower value is correlated with higher profitability (Cretu et al., 2020). This aspect is not always available since the feed price is also variable, depending mainly on its formulation and ingredients' quality.

Therefore, a small decrease in FCR could not reduce the variable cost if the feed price is high. In this case to have a clear image of the

feeding efficiency FCR must be correlated with the cost of the fodder.

The conversion factor values for all experimental groups were within the ranges reported for commercial feed, by other researchers who have conducted similar studies on similar sizes of rainbow trout (Hoseini et al., 2018; Savafi et al., 2019). It was also observed that the feed conversion ratio tends to record higher values with increasing fish weight, registering the best values in small fish groups. Although the mean FCR for various experimental variants differed, no statistically significant differences were found for independent variables represented by density ($P>0.05$). Nevertheless, FCR values for SC1 increased with density while for SC3 the FCR correlated negatively with stocking density (Table 2). If the negative correlation between density and feeding efficiency was found by various authors (Ellis et al., 2002, Boujard et al., 2002) there are also studies reporting no effects on growth or mortality of density until 30 kg/m^3 (Carbonara et al., 2020), 80 kg/m^3 (North et al. 2006) or even 100 kg/m^3 (Boujard et al. 2002). This was also the case in our trial where no significant impact of stocking density was observed within different sizes of fish regardless of the feeding regime. However, the FCR varied significantly (Anova, $P<0.05$) among different class sizes and between feeding regimes but no interaction effect of these factors was detected ($P>0.05$).

Mean specific growth rate (SGR) values over the experimental period ranged between 1.91% BW/day and 2.44% BW/day for the groups fed with a high protein diet and between 1.74%/BW/day and 2.38% BW/day for the groups fed with a low protein diet, varying also with the size of specimens. However, density-linked effects on SGR were detected among size groups rather than for the groups under different feeding regimes (Table 2).

Table 2. Growth performance indices for different sizes of trout fingerlings fed with different diets and reared in open flow-through outdoor system under different densities

			IWG (g/ex)	RGR - (g/kg/d)	SGR (% BW/d)	FCR	PER
HP	**SC1**	**D1**	10.86±0.67	0.14±0.07	2.44±1.19	0.68±0.12	2.96±0.11
		D2	7.54±0.77	0.10±0.08	2.08±1.81	0.81±0.13	2.51±0.11
		D3	6.77±0.81	0.08±0.12	1.91±0.9 7	0.82±0.09	2.43±0.13
	SC2	**D1**	18.20±0.23	0.26±0.07	2.39±0.95	0.64±0.02	3.23±0.15
		D2	17.62±0.44	0.25±0.01	2.21±0.53	0.70±0.06	2.85±0.14
		D3	14.17±0.46	0.20±0.08	2.13±0.98	0.71±0.08	2.60±0.14
	SC3	**D1**	20.46±0.55	0.28±0.09	2.08±1.16	0.88±0.05	1.86±0.15
		D2	19.80±0.29	0.27±0.13	2.07±2.72	0.81±0.10	2.14±0.19
		D3	21.73±0.61	0.30±0.23	2.15±0.69	0.77±0.11	2.30±0.25
LP	**SC1**	**D1**	7.17±1.17	0.06±0.08	2.38±0.12	0.77±0.27	2.89±0.17
		D2	5.18±1.12	0.09±0.05	2.11±0.13	0.84±0.35	2.65±0.55
		D3	4.40±2.98	0.10±0.06	1.74±0.09	0.93±0.13	2.41±0.33
	SC2	**D1**	16.86±1.1	0.21±0.08	2.29±0.11	0.77±0.11	3.12±0.17
		D2	15.70±0.88	0.22±0.10	2.20±1.55	0.79±0.12	2.95±0.34
		D3	14.21±0.78	0.22±0.09	2.12±0.89	0.82±0.15	2.71±1.21
	SC3	**D1**	20.15±0.99	0.29±0.11	2.11±1.34	1.12±0.23	2.77±0.87
		D2	18.79±1.45	0.25±0.08	1.95±0.99	1.02±0.19	2.83±0.12
		D3	18.03±1.23	0.26±0.96	2.11±1.3	0.88±0.77	2.96±0.19
2-Way ANOVA			**p values (α = 0.05)**				
Density			0.817	0.987	0.039*	0.96	0.54
Size			0.002*	0.001*	0.401	0.005*	0.026*
Feed			0.046*	0.266	0.506	0.004*	0.035*
Size*Feed			0.631	0.931	0.991	0.432	0.032*
Density*Feed			0.963	0.880	0.073	0.936	0.985
Size*Density			0.484	0.841	0.01	0.286	0.389

Reduced specific growth rate at high rearing density could be attributed to numerous factors as crowding stress, feed intake, or increased energetic cost of feeding (Boujard et al., 2002; Portz et al., 2006; Naderi et al., 2018; Saulnier et al., 2021). In the present study, we did not observe a reduced feed intake in higher densities. More than that, in the larger fish groups (SC3) the density seemed to not have an adverse effect on SGR (Anova one-way, $P>0.05$). Although an interaction effect of density and size was observed, this was rather associated with SC_1 and SC_2.

The most expensive component in trout feeds is the protein and therefore a reduction in the dietary digestible protein (DP) levels without a negative effect on growth performance could improve protein utilization while reducing nitrogen losses (Hua et al., 2019; Kamalam et al., 2020) this strategy contributing also to a reduction of production cost and increase in profitability.

In our experiment, both factors, diet and fish size, and the interaction between these, contributed significantly to the variation in protein efficiency ratio. However, there were no significant differences among variants held in different stocking density conditions ($P>0.05$).

In this study, the specific growth rate was influenced by the fish size and density interaction while feeding efficiency by the effect of feed (protein level) and fish size interaction. The short duration of the experiment and relatively low densities may have been a factor, and differences may be more pronounced over time. Nevertheless, the study showed the importance of adapting feeding management to the fish size and stocking density to optimize production costs.

However, to have a clear image of the impact induced by the application of such management on the production cost, carrying out additional research is necessary.

CONCLUSIONS

The present study showed that rearing feeding efficiency of rainbow trout in the rearing conditions tested in this study do not seem to be affected by density but by initial size of the fish and the feed that they receive. Higher densities resulted in significantly lower specific growth rate, in special for smaller fish. The diet and fish size as well as the interaction of these factors induced variation in protein efficiency ratio.

ACKNOWLEDGEMENTS

The authors are grateful for the technical support offered by MoRAS through the Grant POSCCE ID 1815. (www.moras.ugal.ro).

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

HELMINTHS AND HELMINTH COMMUNITIES OF *SILURUS GLANIS* (LINNAEUS, 1758) FROM THE TUNDJA RIVER, BULGARIA

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Abstract

Ecologoparasitological research was done based on the helminths and helminth communities of wels catfish (Silurus glanis Linnaeus, 1758) from the freshwater ecosystem of the Tundja, Aegean Water Basin. As a result of the examined seven specimens of wels catfish, three taxa of helminths were found. The dominant structure of the helminth communities was determined. Eustrongylides excisus Jägersiöld, 1909, larvae is a core species for helminth communities of S. glanis (P% = 42.86). S. glanis from the river ecosystem is a new host record for E. excisus. The basic ecological indices of the parasitic populations and communities were determined. The bioindication role of the established parasitic complexes was studied. An assessment of the ecological status of the studied biocenoses was carried out.

Key words*: bioindication, helminth communities, river Tundja, Silurus glanis*.

INTRODUCTION

Tundzha River is the third largest river in Bulgaria (390 km; after the Danube and Iskar rivers) and the Maritsa River's largest tributary, Aegean Water Basin. The river springs from the Balkan Mountains, from 2083 m above sea level. The Tundja River flows into the Maritsa River near Edirne, Turkey, at 32 m above sea level. The waters of the river are used for agriculture, domestic and industrial water supply, electricity, etc. The aquatic ecosystem and its adjacent territories are characterised by great biological diversity, related to the declaration of a number of protected areas and zones. Parasites and parasitic communities reflect the state of the habitat. Most helminths have complex developmental cycles. Therefore, infection indices largely reflect the integrity of food chains, biodiversity, etc. Parasites and parasitic communities have been studied by a number of authors (Margaritov, 1959; Margaritov, 1966; Kakacheva et al., 1978; Soylu, 2005; Goga & Codreanu-Bălcescu, 2013; Kirin & Kuznamova, 2014; Őktener, 2014; Roohi et al., 2014; Abdybekova et al., 2020, etc.). The catfish (*Silurus glanis* Linnaeus, 1758) from the Tundzha River has not been the subject of ecological parasitological research. The study presents data on the endohelminths and helminth communities of catfish (*S. glanis*) from the Tundzha River and discusses the condition of the communities from the studied part of the river.

MATERIALS AND METHODS

In 2019, seven specimens of wels catfish (*Silurus glanis* Linnaeus, 1758) from the Tundja River, Bulgaria, were examined for helminths. According to permission from the Ministry of Agriculture, Food and Forestry of the Republic of Bulgaria, the fish were caught by angling. The scientific name of the fish was present, according to Froese & Pauly (Eds.) (2020). The fish were caught in the section of the river with coordinates: 42°33′12″N, and 25°38′21″ E; 309 m altitude, located between the Balkan Mountain and the Mountain range Sredna Gora, about 20.5 km far away from the town of Kazanlak, Central Southern Bulgaria. The helminthological studies were carried out according to Zashev & Margaritov (1966); Bauer (Ed.) (1987); Moravec (2013). Helminth specimens were fixed in 70% of ethyl alcohol. Species diversity was determined on temporary slides carried out by the method of Moravec (2013) and Petrochenko (1956). Two levels analysed

helminth community structure: on the level of component community (prevalence (P%); mean intensity (MI) for the determined species) and on the level of infracommunity (total number of fish species; total and mean number of fish specimens; Brillouin's diversity index - HB). In the component community, the found species were divided into 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). The obtained results were statistically processed using Statistica 10 (StatSoft Inc., 2011) and MS Exel (Microsoft 2010).

RESULTS AND DISCUSSIONS

Characteristics of the studied fish species

Silurus glanis Linnaeus, 1758 (Siluridae) is a brackish, benthopelagic, non-migratory, heat-loving freshwater fish species. The fish species is naturally distributed in Europe and Asia, including in the Aegean Sea and the Maritsa River Basin. The species inhabits the middle and lower parts of the rivers, reservoirs, etc. It prefers slow-flowing and standing waters with shelters and subterranean. *S. glanis* is a typical predator. Only in the first year, he is eating zooplankton organisms and macrozoobenthos. Foods for adult fish are other species of fish, frogs, waterfowl birds and mammals. Due to plants' swift pace, valued qualities of this fish as food, *S. glanis* is subject to artificial breeding and a species for industrial and sport fishing. The wels catfish is protected by the Berne Convention (Annex 3 - Protected Fauna). IUCN Red List Status of the species is Least Concern (=LC, IUCN) (Froese & Pauly, 2020, Eds.). The species is not protected according to the Republic of Bulgaria's national legislation. Of the studied seven specimens of catfish from the Tindja River, two specimens are free of helminths.

Helminths and helminth community structure

As a result of the ecological-parasitological examinations of 7 specimens of catfish from the Tundja River, infestation with three types of endohelminths was established: *Acanthocephalus lucii* (Müller, 1776) Lühe, 1911; *Eustrongylides excisus* Jägerskiöld, 1909, larvae and *Contracaecum* sp., larvae, belonging to two classes, three orders, three families and three genera (Table 1).

Table 1. Biodiversity and ecological indices of helminths and helminth communities of *Silurus glanis* Linnaeus, 1758 from the Tundja River

Silurus glanis (N[1] = 7) Helminth species	n[2]	p[3]	P%[4]	MI[5]
Class Acanthocephala (Rudolphi, 1808) Skrjabin et Schulz, 1931 **Order** Echinorhynchidae Southwell et Macfie, 1925 **Family** Echinorhynchidae (Cobbold, 1879) Hamann, 1892 **Genus** *Acanthocephalus* Koelreuther, 1771				
Acanthocephalus lucii (Müller, 1776) Lühe, 1911	1	1	14.29	1.0
Class Nematoda Rudolphi, 1808 **Order** Dioctophymida (Skrjabin) Schulz et Gvozdev, 1970 **Family** Dioctophymatidae Castellani et Chalmers, 1910 **Genus** *Eustrongylides* Jägerskiöld, *1909*				
Eustrongylide excises Jägerskiöld, 1909, larvae	3	10	42.86	3.34
Order Ascaridida Skrjabin et Schulz, 1940 **Family** Anisakidae Skrjabin et Karokhin, 1945 **Genus** *Contracaecum* Railliet et Henry, 1912, larvae				
Contracaecum sp., larvae	1	2	14.29	2.0

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.

Ac. lucii parasitises as an adult stage in various species of freshwater fish: Cyprinidae, Percidae, Siluridae, Salmonidae, Esocidae, Gadidae, Cobitidae, Anguillidae. Intermediate hosts of this acanthocephalan species are crustaceans *Asellus aquaticus* (Linnaeus, 1758) (Petrochenko, 1956; Bauer, 1987) (Table 4). Bulgaria, *Ac. lucii* was found as the helminth species of *S. glanis* from the Danube River and *Squalius cephalus* (Linnaeus, 1758) from Iskar and Tundzha rivers (Margaritov, 1959); of *Perca fluviatilis* Linnaeus, 1758 (Margaritov, 1966); of *Ballerus sapa* (Pallas, 1814), *Sq. cephalus*, *Rutilus rutilus* (Linnaeus, 1758), *S. glanis*, *P. fluviatilis*, *Lota lota* (Linnaeus, 1758), *Acerina schraetser* (Linnaeus, 1758), *Benthophilus stellatus* (Sauvage, 1874), *Proterorhinus marmoratus* (Pallas, 1814) (Kakacheva-Avramova et al., 1978); of *Sq. cephalus* (Cakic et al., 2004); of *L. lota* and *Zingel zingel* (Linnaeus, 1766) (Atanasov, 2012); of *Abramis brama* (Linnaeus, 1758) (Chunchukova et al., 2017); of *Alburnus alburnus* (Linnaeus, 1758) (Chunchukova et al., 2018), from the Danube River; of *P. fluviatilis* (Shukerova et al., 2010) and

A. brama (Chunchukova et al. 2016), from the Lake Srebarna; of *R. rutilus* from the Luda Yana River (Kirin et al., 2019); of *Sq. cephalus* from the Ogosta River (Chunchukova et al., 2020), etc. *E. excisus*, larvae are developed with the participation of the first intermediate host oligochaetes (blackworm *Lumbricus variegatus* Linnaeus, 1758, sludge worm *Tubifex tubifex* (Muller, 1774), *Limnodrilus* sp.) and the second fish species, amphibians (Marsh frog, *Pelophylax ridibundus* (Pallas, 1771) (= *Rana ridibunda* Pallas, 1771) and reptiles (Dice snake, *Natrix tesselata* (Laurenti, 1768). The adult nematodes parasitic in the glandular stomach of cormorants [Great Black Cormorant *Phalacrocorax carbo* (Linnaeus, 1758) and Pygmy Cormorant *Microcarbo pygmeus* (Pallas, 1773) (= *Ph. pygmaeus* Pallas, 1773)] (Moravec, 2013) (Table 4).

In Bulgaria, the species is found of *Sander lucioperca* (Linnaeus, 1758) (= *Lucioperca lucioperca* Linnaeus, 1758) (as paratenic host) and of *Gobius* sp. (as intermediate host), of *Aspius aspius* (Linnaeus, 1758) from the Danube River (Kakacheva et al., 1978; Margaritov, 1959); of *P. fluviatilis* from the Zhrebchevo Reservoir (Nedeva & Grupcheva, 1996) and the Srebarna Lake (Shukerova & Kirin, 2007; Shukerova et al., 2010); of *S. glanis*; *L. lota*, *Neogobius melanostomus* (Pallas, 1814) (= *Neogobius cephalarges* Pallas, 1814), *N. kessleri* (Gunther, 1861), *P. fluviatilis* from the Danube River (Atanasov, 2012); of *P. fluviatilis* from the Arda River (Kirin et al., 2013a) from the River Danube and Srebarna lake (Kirin et al., 2013b); of *Rutilus frisii* (Nordmann, 1840) and *Alburnus chalcoides* (Güldenstüdt, 1772) from the Veleka River (Kirin, 2014); of *S. glanis* from the Ivaylovgrad Reservoir (Kirin & Kuzmanova, 2014), etc. *Contacaecum* sp. is reported of *Chondrostoma nasus* (Linnaeus, 1758) and *A. alburnus* from the Danube River (Zaharieva & Zaharieva, 2020a, b; Zaharieva & Kirin, 2020a, b, respectively; Chunchukova et al., 2019), etc. In previous studies, specimens of *Contracaecum* of *S. glanis* were referred to as the species *Contracaecum bidentatum* (Linstow, 1899) (Kakacheva-Avramova, 1977; Kakacheva-Avramova et al., 1978; Kirin & Kuzmanova, 2014) (Table 3).

Component community

The presented helminth taxa were found in 5 of the studied seven catfish specimens (71.43%). Prevalence (P%), mean intensity (MI) and rank were determined for each taxa. *E. excisus* (P% = 42.86) is a core species of the endohelminth communities of *S. glanis* from the Tundja River. The other two species are component (both with P% = 14.29). *E. excisus* is also with the highest mean intensity (MI = 33.34), followed by *Contracaecum* sp. (MI = 2.0). Only one specimen of *Ac. lucii* was fixed in the infected specimen of catfish. *Ac. lucii* is autogenic species. *E. excisus* and *Contracaecum* sp. are allogenic species. The established taxa are generalists for the helminth communities of *S. glanis* from the Tundzha River, Bulgaria (Table 1).

Infracommunity

A total of two examined specimens of *S. glanis* are free of helminths (28.57%). In this study, no mixed invasion was detected. The maximum number of parasites found in a single specimen by the host is four (*E. excisus*). The average number of all endohelminth specimens is low (0.98±0.62), as well as the value of Brillouin's diversity index (HB = 0.45±0.42) (Table 2).

Table 2. Infracommunity data

Number Of helminth species		
Number of infected fish	2	5
Number of helminth species	0	1
Number of helminth specimens		
Total number	13	
Mean ± SD	0.98±0.62	
Range	1-4	
Mean HB ± SD	0.45±0.42	

A total of 13 endohelminth taxa of catfish have been reported in Bulgaria. According to the study, only three taxa were identified (23.08%). Two of the identified species (*A. lucii* and *E. excisus*) have been reported in previous studies as catfish helminths in the country. Detected specimens of the genus *Contraceaceum* have not been identified (Tables 1, 3).

Table 3. Endohelminths of *Silurus glanis* from freshwater ecosystems of Bulgaria

Species diversity	Authors	Freshwater ecosystems (Biotopes)
Trematoda		
Orientocreadium siluri (Bychowski & Dubinina, 1954) Yamaguti, 1964	Kakacheva-Avramova, 1977	river Danube (town (t.) Silistra)
	Kakacheva, Margaritov, Grupcheva, 1978	river Danube (t. Silistra)
	Atanasov, 2012	river Danube (village (v.) Archar, v. Botevo, t. Svishov)
Nicolla skrjabini (=*Crowcrocoecum skrjabini*)	Margaritov, 1966	river Danube
Cestoda		
Triaenophorus nodulosus (Kuperman, 1968)	Atanasov, 2012	river Danube (v. Archar)
Silurotaenia siluri (Batch, 1786) Nybelin, 1942	Margaritov, 1964	river Danube (t. Svishov)
	Kakacheva-Avramova, 1977	river Danube (t. Silistra)
	Kakacheva, Margaritov, Grupcheva, 1978	river Danube (t. Svishov, t. Ruse, t. Silistra)
	Atanasov, 2012	river Danube (v. Archar)
Glanitaenia osculata (=*Proteocephalus osculatus*)	Kabaivanski, 1935	river Danube
	Margaritov, 1959	river Danube (t. Ruse, t. Svishov)
	Margaritov, 1960	lake Shabla
	Kakacheva, Margaritov, Grupcheva, 1978	river Danube (t. Ruse, t. Svishov)
	Kirin, Kuzmanova, 2014	Reservoir Ivaylovgrad
Acanthocephala		
Pomphorhynchus laevis (Müller, 1776)		
	Margaritov, 1966	river Danube
	Kakacheva-Avramova, 1977	river Danube (t. Silistra, t. Svishov, t. Lom)
	Kakacheva, Margaritov, Grupcheva, 1978	river Danube (t. Svishov, t. Ruse, t. Vidin, t. Lom t. Tutrakan)
	Atanasov, 2012	river Danube (v. Archar, v. Dobri dol, t. Svishov, v. Botevo, v. Gomotarci, v. Vardim, v. Novo selo, v. Simeonovo, t. Kozloduj)
Acanthocephalus lucii (Müller, 1776) Lühe, 1911	Margaritov, 1959	river Danube (t. Svishov)
	Kakacheva, Margaritov, Grupcheva, 1978	river Danube (t. Vidin, town Silistra, t. Svishov)
Nematoda		
Eustrongylides excisus (Jägerskiöld, 1909)	Atanasov, 2012	river Danube (v. Archar, v. Dobri dol, v Gomotarci)
	Kirin, Kuzmanova, 2014	Reservoir Ivaylovgrad
Contracaecum bidentatum (Linstow, 1899)	Kakacheva-Avramova, 1977	river Danube (t. Svishov)
	Kakacheva, Margaritov, Grupcheva, 1978	river Danube (t. Ruse, t Vidin, t. Silistra, t. Svishov)
	Kirin, Kuzmanova, 2014	reservoir Ivaylovgrad
Rhabdochona sp. juv	Kakacheva-Avramova, 1965	rivers Maritsa, Asenitsa
Rhabdochona sp.	Margaritov, 1966	river Danube
Rhabdochona sp., larvae	Kakacheva, Margaritov, Grupcheva, 1978	river Danube (t. Vidin t. Lom)
Rhabdochona denudate	Kakacheva-Avramova, 1965	rivers Maritsa (t. Pazardzhik, v. Ognyanovo, v. Kovachevo, t. Septemvri, v. Sadovo, t. Svilengrad), Topolnitsa (v. Srebrino, t. Pazardzhik), Chepinska (v. Kovachevo), Asenitsa (t. Asenovgrad, v. Katunitsa), Sushitsa (v. Bogdantsi), Syuyutlijka (v. Kiril-Antonievo, v. Starozagorski bani), Bedechka (t. Stara Zagora), Harmanlijska (area "Popov bent" and village Bregovo), reservoir "40-te izvora"

Researches on catfish parasites are mainly related to the Danube river basin. In most of them, the parasitic communities are not analysed. Registered helminth taxa have complex development cycles involving more than one host. Catfish is the definitive host only for *Acanthocephalus lucii*. The helminth species inhabit the intestine of the host. *E. excisus* and *Contracaecum* sp. are third stage larves with localisation body cavity/ abdominal cavity and mesentery, respectively. Specified invertebrate intermediate hosts are approved bioindicators for the saprobity in the habitats. *Asellus aquaticus* (Linnaeus, 1758) and *Lumbricus variegatus* Linnaeus, 1758 indicated α-mesosaprobity. *Limnodrillus* sp. indicated p-α-mesosaprobity and *Tubifex tubifex* - p-saprobity. The apparent dominance of *E. excisus* and the indicated bioindicator role of the invertebrate intermediate hosts point to p-α-mesosaprobity in the studied section of the river. The values of the prevalence and mean intensity of *E. excisus* (core species in the study) showed very different values as in the studies from Bulgaria and other countries (Table 4). The presented studies do not establish regularities in the values of prevalence and mean intensity related to the type of freshwater ecosystems – lotics or lentics. It is assumed that the obtained values

are closely related to the intensity of the first intermediate host populations and those of small fish, frogs, reptiles (second intermediate hosts), which are food for the large predator, the catfish.

Table 4. Prevalence and mean intensity of *Eustrongylides excisus* as a helminth species of *Salmo trutta* from freshwater ecosystems in Bulgaria and other countries

Author	Localisation	P%	MI (range)
From Bulgaria			
Atanasov, 2012	river Danube (villages Archar, Dobri dol, Gomotartsi)	6.38	0.15 (3-11)
Kirin, Kuzmanova, 2014	reservoir Ivaylovgrad	27.82	2.0±0.29 (1-2)
Kirin, Chunchukova, 2021 – this study	river Tundzha	42.86	3.34± (2-4)
From other countries			
Soylu, 2005	lake Durusu (Terkos), Turkey	41.8	1.37 (2-28)
Goga & Codreanu-Bălcescu, 2013	lake Viktoria, Romania	10	-
Roohi et al., 2014	Anzali International wetland, Iran	69.77	5.37±4.65 (1-21)
Abdybekova et al., 2020	Kazakhstan	33	54.33 (72-254)

CONCLUSIONS

The study presents the first data on the helminths and helminth communities of the Tundzja River catfish. Of the three found helminth species, *A. lucii* is a core species, and the other two are component species for the helminth communities of *S. glanis*. Only *A. lucii* is an autogenic species in communities. The values of the prevalence and mean intensity are closely related to the intensity of the intermediate host populations and food chains' integrity.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

STRUCTURAL CHARACTERISTICS OF THE SOIL INVERTEBRATE COMMUNITIES FROM TWO FRAGMENTED NATURA 2000 SITES FROM ROMANIA

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Abstract

Wetland ecosystems are dependent of groundwater. They provide goods and ecosystem services. Any anthropic activity will affect their structure. This ecological damage could be revealed by using the biological indicators, as soil invertebrate communities. In 2018, two fragmented Natura 2000 sites were studied: Forest and Eutrophic Marshes from Prejmer (ROSCI0170) and Lempeş Fortress Hill-Hărman Marsh (ROSCI0055). 80 soil samples were investigated, from four fragments in each sites. Two structural parameters were analysed: numerical abundance and constancy. In total, 19 taxa were identified, with 1108 individuals. The highest values of numerical abundances were obtained by the Oribatida mites and Collembola. In Prejmer, 23.52% from the total number of taxa were euconstant, 17.64% constant, 41.17% accessory and 17.64% accidental. The soil fauna from Hărman was represented only by accessory (53.84%) and accidental taxa (46.15%). The dominance of the accidental and accessory taxa demonstrating that the two protected area were not characterized by stable communities. The canonical analysis revealed that the type of habitat influenced the spatial distribution of soil invertebrate communities, defining distinct groups for marsh ecosystems, alluvial forests and deciduous forests.

Key words*: fragment, invertebrate, soil, structure*.

INTRODUCTION

Groundwater dependent ecosystems are natural ecosystems that integrate different components dependent of groundwater (cave and aquifer ecosystems, springs, streams, lakes, rivers, swamps, estuaries and coastal ecosystems, wetlands-swamps, riparian systems, alluvial systems and other terrestrial systems - wetlands, meadows) (Eamus et al., 2006; Kløve et al., 2014). Wetlands come in many different forms. They can be tidal zones, marshes, bogs or swamps among many other types. These types of ecosystems offer to human societies a wide range of essential goods and services (Daily et al., 1997). Wetlands provide several ecosystem services such as reducing erosion, recharging aquifers, flood control, pollution filter, storm and wind buffer, carbon sink and providing habitat for several wildlife species (Eamus et al., 2005). Groundwater dependent ecosystems are often hydrically and ecologically connected to terrestrial ecosystems through transition zones (Tomlinson & Boulton, 2010). Therefore, we consider that an important component of the biodiversity of terrestrial ecosystems dependent on groundwater, but also on the surface water (as wetlands-swamps), is represented by the soil (edaphic) fauna. In Europe, several biological indicators were used, which were based on groups of organisms (simple indicators) or on whole community of soil fauna (compound indicators). Over the decades, different groups of edaphic invertebrates have been used as bioindicators of natural or anthropogenic ecosystems (Collembola, Nematoda, Acari, Chilopoda, Diplopoda, Protura, Isopoda, Diplura, Coleoptera, Mollusca, etc.). Any anthropic impact (as ecosystem fragmentation) will reflect into modification on structure and functions of soil invertebrates communities (Lavelle & Bignell, 1997; Ruf, 1998; Ponge et al., 2003; Sanchez-Moreno & Navas, 2007; Bedano et al., 2011; Santamaria et al., 2012; Skubała & Zaleski, 2012; Manu et al., 2019).

In this context, the present paper aims to highlight the structural characteristics of the

soil invertebrate communities from two main hypotheses: the investigated ecosystems were characterized by stable edaphic fauna and how the type of vegetation habitats influenced the structure of these communities?

MATERIALS AND METHODS

The study area

The present study was made in November 2018, in two fragmented Natura 2000 sites from Braşov County, Romania: Forest and Eutrophic Marshes from Prejmer (ROSCI0170) and Lempeş Fortress Hill-Hărman Marsh (ROSCI0055). The nature reserve Forest and Eutrophic Marshes from Prejmer, with an area of 345 hectares, was declared as protected area by law no. 5 of March 6, 2000, published in the Official Monitor of Romania, no. 152 of April 12/2000. In this area there are terrestrial ecosystems (forests, shrubs, meadows) and freshwater aquatic ecosystems (swamps). Lempeş Fortress Hill-Hărman Marsh has an area of 374 hectares, and was declared as protected area in 2000, by the same law as above.

The ecological investigations were made in four fragments, in each protected area. These were codified as following: PF1 (the first fragment from Prejmer); PF2 (the second fragment from Prejmer), PF3 (the third fragment from Prejmer); PF4 (the fourth fragment from Prejmer): HF1 (the first fragment from Hărman); HF2 (the second fragment from Hărman), HF3 (the third fragment from Hărman); HF4 (the fourth fragment from Hărman) (Figure 1).

Figure 1. Geographical location of fragments from Forest and Eutrophic Marshes from Prejmer (ROSCI0170) (yellow color) and Lempeş Fortress Hill-Hărman Marsh (ROSCI0055) (green color) ecosystems, from Romania, 2018

fragmented Natura 2000 sites, proposing two In the Forest and Eutrophic Marshes from Prejmer**,** the samples from PF1 were located at $45^0$43'46.04"N and $25^0$44'09.03"E; at 514 m altitude. PF2 was located at $45^0$44'52.22"N and $25^0$43'48.66"E; at 503 m altitude. In PF3, the soil samples were taken from $45^0$44'56.90"N and $25^0$42'08.94"E, 508 m altitude. PF4 was situated at $45^0$44'55.46"N and $25^0$41'15.64"E and 501 m altitude. The PF1 fragment was characterized by the habitat 7210 * calcareous fens with *Cladium mariscus*. The rest of fragments were characterized by the following type of habitat: 91 EO alluvial forests with *Alnus glutinosa* and *Fraxinus excelsior* (*Alno-Padion*, *Alnion incanae*, *Salicion albae*).

The fragments from Lempeş Fortress Hill-Hărman Marsh were located and described as following: HF1 at $45^0$43'03.63"N and $25^0$40'03.61"E, 514 m altitude, habitat type: 7210 * calcareous fens with *Cladium mariscus*; HF2 at $45^0$43'07.30"N and $25^0$40'00.08"E, 498 m altitude, habitat type: 9170 Galio-Carpinetum oak-hornbeam forests; HF3 at $45^0$44'12.63"N and $25^0$40'27.07"E, 511 m altitude, habitat type: 9170 Galio-Carpinetum oak-hornbeam forests; HF4 at $45^0$43'07.30"N and $25^0$40'00.08"E, 498 m altitude, habitat type: 9130 Asperulo-Fagetum beech forests.

Soil samples

The investigated area in each swamp was by 500 square meters. In total 80 cores (40 samples in each protected area) were sampled for soil fauna, to a depth of 10 cm with a MacFadyen corer, by 5 cm diameter. The samples were taken randomly. The fauna were extracted with a modified Berlese-Tullgren funnel, in ethyl alcohol. The published identification keys were used (Dindal, 1990; Orgiazzi et al., 2016; Krantz, 2009).

Data analysis

The constancy was obtained using the formula: C = 100*pA/P, where: pA - number of samples with taxa A; P - total number of samples. The taxons were classified in four constancy classes: euconstant taxa having constancy of 75.1-100% (C4), constant taxa having constancy of 50.1-75% (C3), accessory taxa having constancy of 25.1-50% (C2) and

accidental taxa having constancy of 1-25% (C1) (Selvin & Vacca, 2004).

The correspondence analysis (CA) between identified taxonomical groups and analysed fragments from the two protected areas; the individual rarefaction were calculated using the BioDiversity Pro 2.0 software, PAST (Hammer et al., 2001).

RESULTS AND DISCUSSIONS

Taking into consideration the taxonomical spectrum of two investigated Natura 2000 sites, we identified in total 19 taxa, with 1108 individuals. These were grouped in seven taxonomic classes: Clitellata, Diplopoda, Chilopoda, Entognatha, Insecta, Arachnida and Gastropoda (Table 1). The highest values of numerical abundances were obtained by the taxa from Oribatida suborder (mites), with 411 individuals and Collembola order (springtails), with 308 individuals. On the opposite there are taxa from Chilopoda, with a total of 4 individuals. Making a comparison between the two investigated fragmented protected areas, we observed that in Forest and Eutrophic Marshes from Prejmer, the both structural parameters recorded higher values (17 taxa and 655 individuals), in comparison with Lempeş Fortress Hill-Hărman Marsh (13 taxa with 453 individuals). In each area, the Oribatida mites and Collembola taxa were numerical dominant (411 individuals and respectively 308 individuals), instead Chilopoda was less represented (4 individuals). From all 17 identified taxa, 58.82% were common for both protected areas, 41.17% were characteristics for Prejmer and only 17.64% from Hărman. In the scientific world is well known that soil invertebrates constitute a valuable bioindicator tool (Gardi et al., 2009; Manu et al., 2019). The obtained data are comparable with other studies from all over the world, which revealed that Collembola, Enchtreidae, Oribatida and Mesostigmata were the most abundant taxa in wetlands (Plum, 2005; Reynolds at al., 2007; Huhta et al., 2011). If we compare the obtained results from Romanian marshes with other types of ecosystems, at international level, we observed that the number of taxon is higher than that from forest ecosystems (9-14 taxa), shrubs (9-11 taxa), arable land (6-12 taxa) or grasslands (16 taxa) (Parisi et al., 2005; Yan et al., 2012). According to these studies, species numbers and abundances of Lumbricidae, Isopoda, Chilopoda and Diplopoda tended to be lower in frequently and/or extensively flooded sites. In bogs, even when they are waterlogged the entire year, species numbers are distinctly higher than the most frequently flooded sites (Plum, 2005; Sterzyńska et al., 2015). At national level these types of studies, which take into consideration the functional groups of invertebrates, are few (Manu et al., 2020). Analysing this literature, we observed that the number of taxa and the numerical abundance of the taxons from the two fragmented marshes recorded lower values, in comparison with those obtained in a protected area "Springs Complex of Corbii Ciungi", characterized by the meadows and riverine scrub habitats (34 functional groups, with 4180 individuals) (Manu et al., 2020).

If we put into discussion the constancy index, quantified for each invertebrate communities from the two investigated protected areas, the study revealed that in Prejmer, 23.52% from the total number of taxa were euconstant, 17.64% constant, 41.17% accessory and 17.64% accidental. On the other hand, the soil fauna from Hărman was represented only by accessory (53.84%) and accidental taxa (46.15%) (Table 1). The dominance of accessory and accidental species (with few exceptions (Collembola, Oribatida, Opiidae and Mesostigmata) revealed the fact that the rest of invertebrates communities are only occasional present in investigated fragments of the two areas. We could suppose that the fragmentation of the investigated ecosystems impact the soil invertebrate communities, being known that the taxons as Chilopoda, Isopoda, Coleoptera, etc., were identified in optimal conditions, in alluvial forest (Herlitzius, 1987; Manu et al., 2013; Kolesnikova et al., 2016).

Table 1. The structural parameters (numerical abundance and constancy) of identified taxa from Forest and Eutrophic Marshes from Prejmer (ROSCI0170) and Lempeş Fortress Hill-Hărman Marsh (ROSCI0055), Romania, 2018

Taxa	PF1	PF2	PF3	PF4	Total PF	HF1	HF2	HF3	HF4	Total HF
Phylum Annelida										
Class Clitellata										
Subclass Oligochaeta										
Order Haplotaxida										
Family Lumbricidae - Lum	2/ac	4/ac		3/ac	9/ct	2/ac				2/ac
Family Enchytreidae - Enc	13/as	6/as	2/as	1/as	22/eu	5/as	2/ac			7/ac
Phylum Arthropoda										
Subphylum Myriapoda										
Class Diplopoda - Dip	3/ac	1/ac			4/as					
Class Chilopoda - Chi	2/ac		1/ac		3/as		1/ac			1/ac
Subphylum Crustacea										
Subphylum Hexapoda										
Class Entognatha										
Order Collembola- Collem	57/ct	83/eu	37/as	29/as	206/eu	48/eu	34/as	10/ac	10/ac	102/as
Order Diplura - Dip	6/ac				6/ac					
Order Protura - Pro	1/ac				1/ac					
Class Insecta										
Order Coleoptera - Col	1/ac				1/ac					
Order Psocoptera - Pso				1/ac	1/ac					
Order Hymenoptera										
Superfamily Formicoidea- For							20/ac			20/ac
Insect larvae- Ins.larv	20/ct	3/ac			23/ac		1/ac	1/ac	1/ac	3/as
Subphylum Chelicerata										
Class Arahnida										
Order Opiliones-Opi		1/ac			1/ac					
Supraorder Acariformes										
Order Trombidiformes										
Suborder Prostigmata Family Trombidiidae- Tro	1/ac			2/ac	3/ac					
Family Bdellidae- Bde	2/ac	4/as		2/ac	8/ct		1/ac	12/as	1/ac	14/as
Order Sarcoptiformes Suborder Oribatida-Ori	140/eu	54/eu	15/ct	17/as	226/eu	113/eu	22/ct	47/eu	3/eu	185/as
Family Opiidae- Opi	32/as	33/ct	2/ac	2/ac	69/eu	8/ac	3/ac	29/as	2/ac	42/as
Suborder Astigmata- Ast								6/ac	2/ac	8/ac
Family Acaridae- Aca	6/as	8/as			14/as	29/eu	1/ac	13/as	4/ac	47/as
Order Mesostigmata- Mes	27/ct	15/as		16/ac	58/ct	14/ct	1/ac	4/as	2/ac	21/as
Phylum Mollusca Class Gastropoda- Gas						1/ac				1/ac
Total no of taxa	15	11	5	9	17	8	10	8	8	13
Total no of individuals	313	212	57	73	655	220	86	122	25	453
Total no of euconstant species (eu)	1	2			4	3		1	1	
Total no of constant species (ct)	3	1	1		3	1	1			
Total no of accesory species (as)	3	4	2	3	7	1	8	3		7
Total no of accidental species (ac)	8	4	2	6	3	3	1	4	7	6

Making an analysis in spatial dynamics of soil invertebrate communities, in each studied area, we observed differences for each studied fragments, especially in Prejmer. The highest numbers of identified taxa and of numerical abundances, in the Prejmer protected area, were obtained in PF1 and PF2, in comparison with PF3 and PF4. This fact was highlighted by the individual rarefaction analysis (Figure 2). Common taxa for each fragment were the following taxa: Enchytreidae, Collembola, Oribatida and Oppiidae, with represent 23.52% from the total identified taxons. In the same time, the first fragments were the only ones, which were characterized by few euconstant (Oribatida and Collembola) and constant taxa (Opiidae, Mesostigmata, insect larvae). The second fragments PF3 and PF4, were dominated by the accessory and accidental species (Table 1).

In Lempeş Fortress Hill-Hărman Marsh, the differences between the four transects are not so evident. In HF1, HF3, HF4 the number of taxa was the same, only in HF2 this parameter recorded the highest value (Table 1; Figure 3). If we put into discussion the numerical abundance, in the HF1 and HF3 were recorded the highest values, in comparison with the other two fragments HF2 and HF4. 38.46% were common taxa for the four fragments from Hărman ecosystems, as following: Collembola, Oribatida, Oppiidae, Acarida and Mesostigmata. Considering the constancy index, the fragments were better represented by accessory and accidental taxa, than euconstant-constant taxons (as Collembola, Oribatida and Acarida) (Table 1).

In order to demonstrate if the types of habitat influence the spatial distribution of soil invertebrate communities, the correspondence analysis (CA) between identified taxonomical groups and investigated fragments was analysed. In Prejmer protected area, four groups were defined: soil invertebrates communities characteristics for PF1: Coleoptera and insect larvae; for PF2 and PF3: Collembola, Opiidae; for PF4: as Psocoptera, Trombidiidae, Bdellidae, Mesostigmata and Lumbricidae (Figure 4).

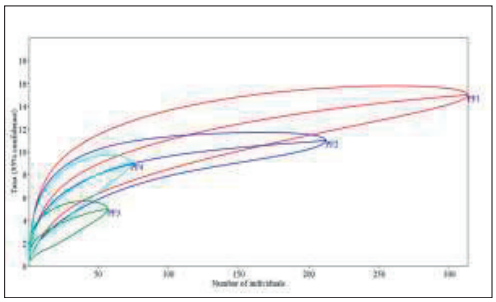

Figure 2. The individual rarefaction of the soil invertebrate communities from Forest and Eutrophic Marshes from Prejmer (ROSCI0170), Romania, 2018

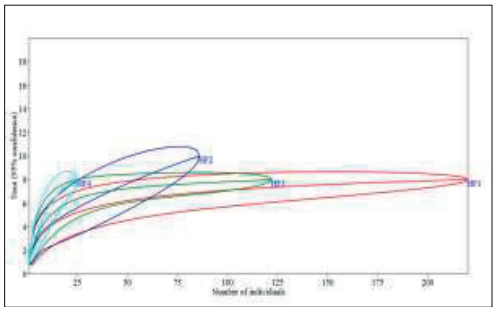

Figure 3. The individual rarefaction of the soil invertebrate communities from Lempeş Fortress Hill-Hărman Marsh (ROSCI0055), Romania, 2018

Even if the PF1 was defined by the calcareous marsh habitat, it is possible that due to the environmental conditions (dryness period), there weren't favorable conditions for development of soil invertebrates communities. On the other hand, the alluvial forest with *Alnus glutinosa* and *Fraxinus excelsior* constituted a proper habitat for the majority of the soil functional groups.

In Lempeş Fortress Hill-Hărman Marsh protected areas, we discovered that the spatial distribution of the soil invertebrate communities was influenced by the type of habitat, describing four groups. The first group HF1 (alkaline marsh) was the characterized by the following taxa: Lumbricidae, Mesostigmata, Acaridae and Oribatidae; HF2 (oak-hornbeam forest) another distinct group, contains the following taxa as: Chilopoda, Formicoidea, insect larvae; HF3 and HF4 offered proper habitats (oak-hornbeam forest and beech forest) for mites' taxa, Opiidae, Bdellidae and Astigmata.

We observed that in the two protected areas, the soil invertebrate communities formed a distinct group in marsh ecosystems, possible due to the specifically environmental conditions.

International ecological researches concerning the influence of the type of habitat on the structure of soil invertebrates' communities were well developed in Europe. These studies revealed that there are specifically environmental conditions (taking into account the abiotic and biotic factors) for each investigated habitats, which influenced directly or indirectly the soil invertebrate fauna (Plum, 2005; Manu, 2013; Skubala & Zaleski, 2012; Sterzynska et al., 2015; Manu et al., 2020).

The results of our study is in concordance with those from Europe.

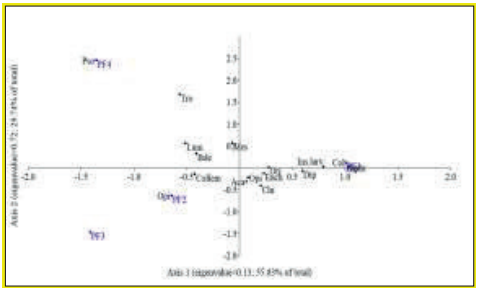

Figure 4. Correspondence analysis (CA) between identified taxonomical groups and analysed fragments from Forest and Eutrophic Marshes from Prejmer (ROSCI0170), Romania, 2018 (the short names of the taxa are mentioned in Table 1)

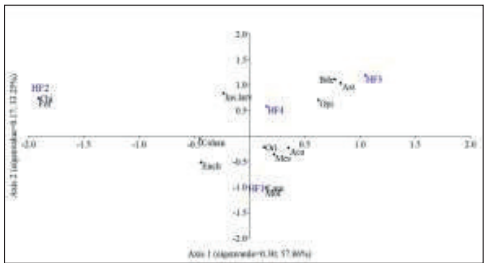

Figure 5. Correspondence analysis (CA) between identified taxonomical groups and analysed fragments from Lempeş Fortress Hill-Hărman Marsh (ROSCI0055), Romania, 2018 (the short names of the taxa are mentioned in Table 1)

CONCLUSIONS

In oder to highlight the structure characteristic of the soil invertebrate fauna from two fragmented Natura 2000 sites (Forest and Eutrophic Marshes from Prejmer - ROSCI0170 and Lempeş Fortress Hill-Hărman Marsh - ROSCI0055), 80 soil samples were analysed, from four fragment/each protected areas, in 2018. Two main structural parameters analysed were numerical abundance and constancy. In total, 19 taxa were identified, with 1108 individuals. The highest values of numerical abundances were obtained by the Oribatida mites and Collembola. On the opposite there are taxa from Chilopoda. In both protected areas the dominant taxa were accessory and accidental ones, only in Prejmer were identified euconstant and constant functional groups, but there are poorly represented. These data revealed that the analysed fragmented areas are not characterized by stable soil invertebrate communities. Using the correspondence analysis, we demonstrated that the type of habitat influenced the spatial distribution of soil invertebrate communities, defining distinct groups for marsh ecosystems, alluvial forests and deciduous forests.

ACKNOWLEGMENTS

This study was funded through project PED number 453/2020 code PN-III-CERC-CO-PED-2-2019, supported by Executive Unit for Financing Higher Education, Research, Development and Innovation, for Romania (UEFISCDI). The theme of the article is according to the general framework of the project number RO1567-IBB01/2021 of the Institute of Biology Bucharest of the Romanian Academy.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

EVALUATION OF CYPRINIDS CONDITION REARED IN TWO INTEGRATED MULTI-TROPHIC AQUACULTURE (IMTA) SYSTEMS BASED ON A FEW SOMATIC INDICES (VSI, HSI, GaSI AND RGL)

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Abstract

The present study was carried out to measures and analyses the condition of cyprinids, reared in a two IMTA systems by using a few organosomatic indices. The first pond (PCP) was used for rearing common carp in polyculture with other cyprinids. The second pond was divided in two parts: first part-common carp (CP) and the second part - polyculture (PP). During this experimental period (May to September), the biometric measurement was made monthly. Regarding to the feeding regime, fish were feed only in PCP and CP pond (part of CP-PP pond). At the end of the experiment the results showed an increase in viscerosomatic index (VSI), hepatosomatic index (HIS), gastrosomatic index (GaSI) at cyprinids in CP and PCP. This is mainly due to the fact that only in CP and PCP feed was administered. An increase in the relative gut length (RGL) index was observed especially in grass carp, followed by the other cyprinids in the PP pond, part of the CP-PP pond, in which feed was not administered. In conclusion, this shows us that cyprinids can adapt to the natural feeding conditions in case of feed absence (cereals mix).

Key words*: cyprinids, IMTA, organosomatic indices, polyculture, relative gut length.*

INTRODUCTION

Over the past few decades, the increasing demand for world fishery production has led to a significant expansion of aquaculture, which accounts for about half of global seafood production (Zhu et al., 2019).

Impact of common carp (*Cyprinus carpio*, Linnaeus 1758) pond production on fish pond ecosystems has been extensively studied in Central and Eastern Europe (Pechar et al., 2002). Currently, the evaluation of possible positive, as well as negative effects of fishery management on surface water quality (Vsetickova et al., 2012) is another important issue, often linked to integrated aquaculture systems.

Strategies that aimed to reduce the impact of nutrients on aquaculture effluents have focused on optimizing feed composition, improving feed and feeding technology, as well as feeding strategy (Boyd, 1998).

Integrated multi-trophic aquaculture (IMTA) is the farming, in proximity, of species from different trophic levels and with complementary ecosystem functions in a way that allows one species uneaten feed and wastes, nutrients and by products to be recaptured and converted into fertilizer, feed and energy for the other crops, and to take advantage of synergistic interactions among species while biomitigation takes place (Chopin, 2013). The multi-trophic sub-systems are integrated in IMTA that refers to the more intensive cultivation of the different species in proximity of each other, linked by nutrient and energy transfer through water (Sasikumar & Viji, 2016).

Cyprinidae polyculture is also favored, based on the assumption that each fish species has its own feeding niche that does not overlap much

with the feeding niche of the other species. As a result, a large fraction of natural food available in the pond is used in multi-species systems (Khan et al., 2016).

Feed and feeding are the key components of cost-effective aqua-culture, economic and nutritional achievements of the aquaculture mainly depend upon supplementary diets (Omosowone & Ogunrinde, 2018).

Fish diet has been found to be an important factor governing fish growth, welfare, condition factor, fecundity and migration patterns (Adeyemi et al., 2009; Rao, 1974). Feeding is the dominant activity of the entire life cycle of fish (Joadder & Hossain, 2008). The study of the food and feeding habit of fishes provide keys for the selection of culturable species and such information is necessary for successful fish farming (Manon & Hossain, 2011).

The study of fish condition is usually based on the analysis of length-weight data and of other indices like organosomatic indicators. Organosomatic indices can be described as the ratios of organs to body weight when the measured organ in relation to body mass can be directly linked to some environmental changes (Ronald & Bruce, 1990). It is manifested through changes in size that are reflected through a reduction or increase, influenced by environmental factors. Size and weight of the organs are related to the overall length and weight of fish and indicate the general status of health of the fish (Ronald & Bruce, 1990). However, organosomatic indices may provide more specific information related to the function of the selected organ (Martin-Diaz et al., 2005). It can also be used as indices of changes in nutritional and energy status (Maxwell & Dutta, 2005).

The aim of this research was to evaluate the condition of cyprinids, reared in a two IMTA systems, by using a few organosomatic indices like viscerosomatic index, hepatosomatic index, gastrosomatic index and relative gut length.

MATERIALS AND METHODS

Description of the study sites. The research were conducted at the "S.C. Piscicola Iasi" fish farm, which is situated at 24 km from Iasi, more exactly in the Larga Jijia village. The water source is represented by Jijia river. Both inlet and outlet are made gravitationally, by using monk hydraulic constructions. The location of the farm is described in the work published by Petrea et al. (2017).

Design experimental. The experiment was performed in two ponds with an area of 0.45 ha each, with an average water depth of 1.5 m.

The first pond (PCP) was used for rearing carps in polyculture like as: common carp (*Cyprinus carpio* - 2500 exemplars) with grass carp (*Ctenopharyngodon idella* - 100 exemplars), bighead carp (*Hypophthalmichthys nobilis* - 40 exemplars) and silver carp (*Hypophthalmichthys molitrix* - 40 exemplars).

The second pond was divided by using a net, and stocked as follows: first part with an area of 0.15 ha (CP - with 2000 common carp exemplars) and the second part with an area of 0.30 ha (PP - with 500 common carp exemplars, 40 silver carp exemplars, 40 bighead exemplars and 100 grass carp exemplars).

At the beginning of the research the individual average of common carp biomass weight was 61.9±10.0 g/ex, for silver carp was 2025.1±248.9 g/ex, for bighead carp was 1880.5±193.3 g/ex and for grass carp was 199.9±19.7 g/ex.

This experimental design was used for a growth research that had lasted from May to September characteristic for a carp growth cycle.

In the second pond, CP-PP pond, an intermittent hydraulic regime was applied during the day-time, in order to transport the fish metabolic wastes from CP pond area, to PP pond area, in order to assure the development of phytoplankton and therefore, to generate a better wastes management and valorisation.

Regarding to the feeding regime, fish were feed only in PCP and CP pond (part of CP-PP pond). The administered feed had a crude protein content of 28% and was represented by a mix of cereals (wheat lees, dry maize dregs, sunflower groats) in equal amounts and flour protein. Feed was manually administered twice/day, only in PCP and CP, for five days/week.

During the research, fisheries control were carried out every month. At each fishing control, biometric measurements were

performed and also, fish were retained for the organosomatic analysis.

Organosomatic analysis. The analysis of organosomatic indices was determined at the Research Laboratory of Food Science, Food Engineering, Biotechnologies and Aquaculture Department from "Dunărea de Jos" University of Galaţi. Until the laboratory, during the transportation the fish were kept in refrigerated boxes. The fishes from sampling ponds were wiped dry with the help of a cotton towel. Each exemplar was weighed on a electronic balance. Then was followed immediately by fish evisceration to determine the organosomatic indices. Complete care was taken with the gut to prevent either the injury or pressure to avoid the loss of gut contents. Total length and total weight of gut was also recorded. Among the determined indices are listed: viscerosomatic index (VSI), hepatosomatic index (HSI), gastrosomatic index (GaSI) and relative gut length (RGL). These indices were calculated using the following formulas (sources 4, 7):

VSI (%) = 100 x viscer weight (g)/weight of fish (g) (1)

HSI (%) = 100 x liver weight (g)/weight of fish (g) (2)

GaSI (%) = 100 x weight of full gut (g)/ weight of fish (g) (3)

RGL = gut length (cm)/total length of fish (cm) (4)

Statistical analysis

The results obtained in this research were statistically analysed using IBM SPSS Statistics 20.0, Microsoft Excell 2010. To determine significant differences among groups was used the one-way analysis of variance (ANOVA); $p<0.05$ was considered as significant.

RESULTS AND DISCUSSIONS

Integrated multi-trophic aquaculture (IMTA) is considered a solution for converting the waste products from one food production process (in this case, fish production) into a source of food for other organisms, generating therefore valuable products, thus, improving the aquaculture industry sustainability and profitability (Petrea et al., 2019).

The individual length-individual weight linear regression.

A. PCP experimental variant

At the beginning of the experimental period, no significant differences ($p>0.05$) were observed between the experimental variants in terms of fish, individual length and individual weight. Therefore, the homogeneity of fish experimental biomass was statistically verified (Levene Test, $p>0.05$).

Common carp. The individual length-individual weight linear regression shows high homogeneity both at the beginning and at the end, but also during the experimental period. Therefore, the following linear regression factors were recorded: R^2 initial = 0.82; R^2 Int.1 = 0.953; R^2 Int.2 = 0.819; R^2 Int.3 = 0.909; R^2 final = 0.768 (Figure 1).

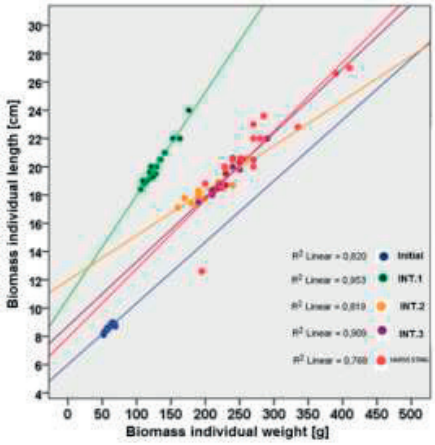

Figure 1. Linear regression of common carp biomass individual length-weight during the experimental period

Silver carp. The individual length-individual weight linear regression shows high homogeneity at the initial, Int.3 and at the final harvesting. Therefore, the following linear regression factors were recorded: R^2 initial = 0.879; R^2 Int.2 = 0.032; R^2 Int.3 = 0.997; R^2 final = 0.817 (Figure 2). The lowest value of linear regression factor, recorded at Int.2 is due to small number of silver carp caught at the control harvesting, this fact conducted to an inconclusive statistically result.

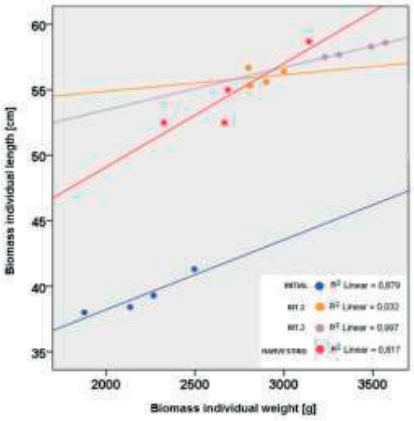

Figure 2. Linear regression of silver carp biomass individual length-weight during the experimental period

Bighead carp. The individual length-individual weight linear regression shows high homogeneity at the initial, Int.1 and at the Int.2 harvesting. Therefore, the following linear regression factors were recorded: R^2 initial = 0.905; R^2 Int.1 = 0.979; R^2 Int.2 = 0.856; R^2 Int.3 = 0.670; R^2 final = 0.523 (Figure 3). The lowest values of linear regression factor, recorded at Int.3 and final harvesting showed that there is an inconsistent linear relation between weight and length growth in the second half of the experimental period (Figure 3).

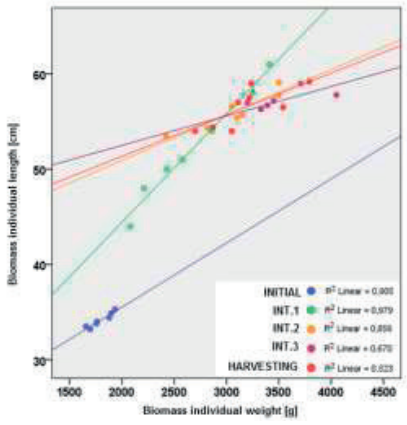

Figure 3. Linear regression of bighead carp biomass individual length-weight during the experimental period

Grass carp. The individual length-individual weight linear regression shows high homogeneity at the initial, Int.1, Int.2 and at the final harvesting. Therefore, the following linear regression factors were recorded: R^2 initial = 0.965; R^2 Int.1 = 0.978; R^2 Int.2 = 0.938; R^2 Int.3 = 0.746; R^2 final = 0.981 (Figure 4).

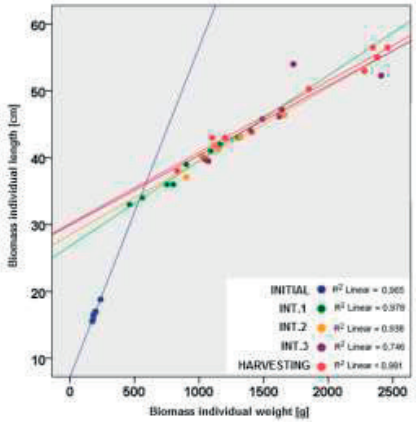

Figure 4. Linear regression of grass carp biomass individual length-weight during the experimental period

B. CP-PP experimental variant

Common Carp - CP. The individual length-individual weight linear regression shows high homogeneity throughout the experimental period. Therefore, the following linear regression factors were recorded: R^2 initial = 0.799; R^2 Int.1 = 0.998; R^2 Int.2 = 0.978; R^2 Int.3 = 0.962; R^2 final = 0.710 (Figure 5).

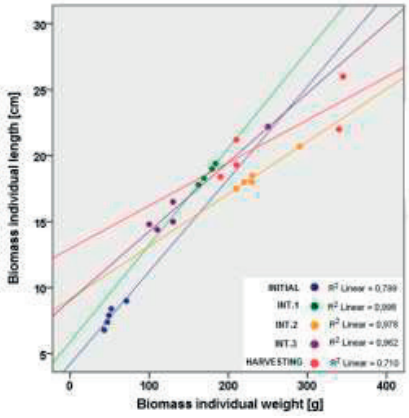

Figure 5. Linear regression of common carp biomass individual length-weight during the experimental period

Common Carp - PP. The individual length-individual weight linear regression shows high homogeneity from the beginning of the experimental period, until Int.3 (Figure 6). Therefore, the following linear regression

factors were recorded: R^2 initial = 0.808; R^2 Int.1 = 0.938; R^2 Int.2 = 0.934; R^2 Int.3 = 0.695; R^2 final = 0.702 (Figure 6).

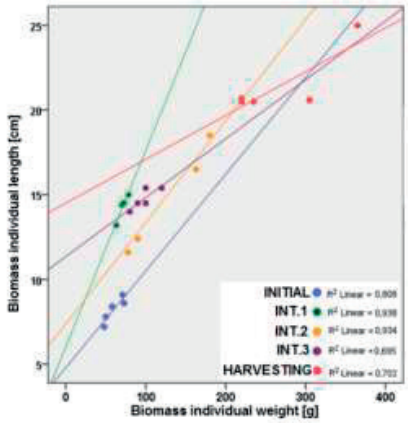

Figure 6. Linear regression of common carp biomass individual length-weight during the experimental period

Silver Carp - PP.

A high homogeneity of the initial and final fish biomass is also highlighted by the individual length-individual weight linear regression. Therefore, the following linear regression factors were recorded: R^2 initial = 0.992; R^2 final = 0.932 (Figure 7).

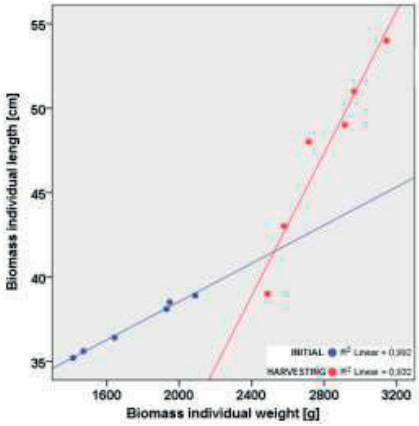

Figure 7. Linear regression of silver carp biomass individual length-weight during the experimental period

Bighead carp - PP. The individual length-individual weight linear regression shows high homogeneity at the initial and Int.2 harvesting. Therefore, the following linear regression factors were recorded: R^2 initial = 0.910; R^2 Int.2 = 896; R^2 Int.3 = 0.692; R^2 final = 0.694 (Figure 8). The lowest values of linear regression factor, recorded at Int.3 and final harvesting showed that there is an inconsistent linear relation between weight and length growth in the second half of the experimental period (Figure 8).

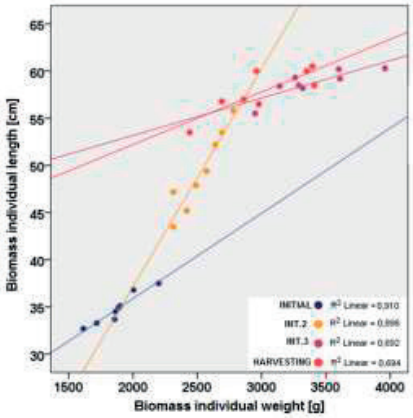

Figure 8. Linear regression of bighead carp biomass individual length-weight during the experimental period

Grass carp - PP. The individual length-individual weight linear regression shows high homogeneity of grass carp biomass during the entire experimental period (Figure 9).

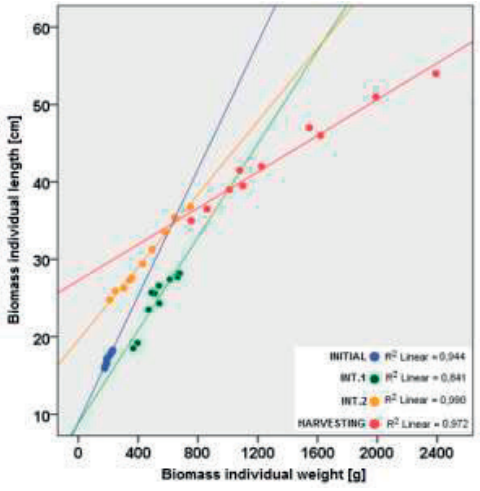

Figure 9. Linear regression of grass carp biomass individual length-weight during the experimental period

Therefore, the following linear regression factors were recorded: R^2 initial = 0.944; R^2 Int.1 = 0.841; R^2 Int.2 = 0.990; R^2 final = 0.972.

Organosomatic indices.

A. Viscerosomatic index (VSI %)

In addition knowledge of some quantitative aspects in fishes is an important tool for the study of biological fundamentals such as viscerosomatic and hepatosomatic indices, because measurement and analysis of these indices are very important in assessing food value (Ighwela et al., 2014).

When comparing the results obtained during the entire production cycle, it is found that VSI index recorded significant higher values ($p<0.05$) in CP pond (14.07±0.93%) in case of common carp, followed by the values obtained in PP pond (11.73±2.14%), respectively in PCP pond (11.40±1.25%) (Figure 10).

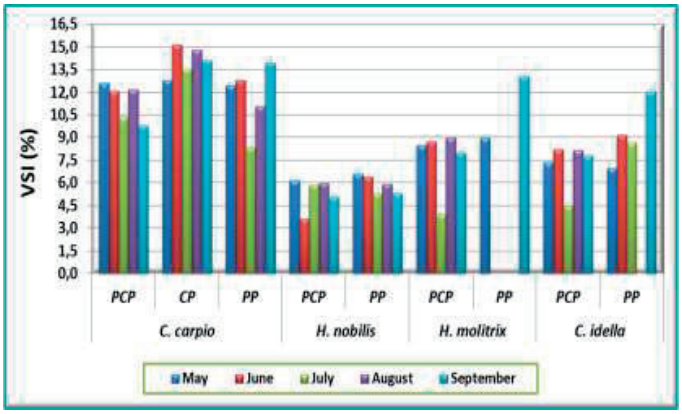

Figure 10. Changes of the viscerosomatic index (VSI) in response to different feeding regime

At the same time, in the case of the other species, it is observed that the VSI index showed higher values in PP pond (bighead carp - 5.90±0.69%, silver carp - 11.00±2.89%, grass carp - 9.21±2.10%), compared to the PCP pond (bighead carp - 5.34±1.05%, silver carp - 7.63±2.11%, grass carp - 7.19±1.55%), in which the feed was administered. No results are available during the experimental period, at intermediary stages (from June to August), because no exemplars of silver carp were harvested.

B. Hepatosomatic index (HSI %)

The study of viscerosomatic and hepatosomatic indices has an important role in the metabolism of the fishes, related to digestion and absorption, synthesis and secretion of digestive enzymes and carbohydrate metabolism (McLaughlin, 1983).

Singh and Canario (2004) observed that hepatosomatic index is one of the most investigated biomarker due to important role of liver in detoxification of pollutants.

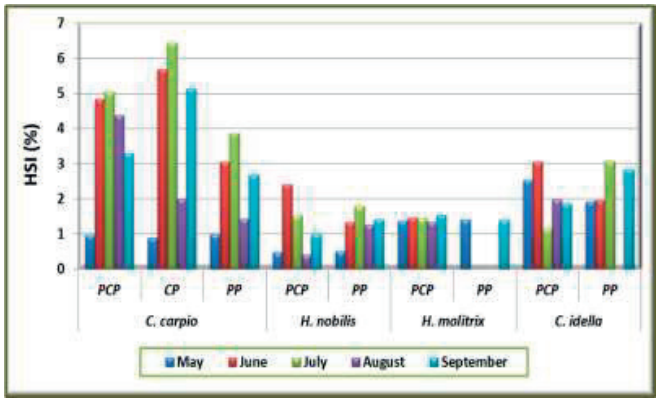

Figure 11. Changes of the hepatosomatic index (HSI) in response to different feeding regime

If at the VSI level there were significant differences only in carps reared in CP pond compared to that raised in PP pond and PCP pond, in case of the hepatosomatic index there were no significant differences ($p>0.05$) in any of the carp species reared in those two systems (PCP and CP-PP) (Figure 11).

During the entire production cycle the mean highest values of HSI were registered for common carp in CP pond (4.03±2.44%), for bighead carp and grass carp in PP pond (1.26±0.47%, respectively 2.45±0.59%) and for silver carp in PCP pond (1.44±0.08%).

Gastrosomatic index (GaSI %)

The gastro-somatic index (GaSI) was used to determine the feeding intensity of fish (Kurbah & Bhuyan, 2018).

The results of GaSI index are presented in Figure 12. Regarding to the results obtained at the level of GaSI index a significant differences ($p<0.05$) between PCP and PP pond was registered in case of grass carp. The highest mean value of GaSI were recorded for common carp in CP-PP pond (4.24±0.44% - CP, respectively 3.31±0.90% - PP) and for bighead carp (2.21±0.81%), silver carp (1.98±0.47%, respectively for grass carp (4.47±0.86%) in PP pond.

The higher values of GaSI obtained in PP part of CP-PP pond may be due to the gravitational current of water (the water supply of the pond is made near to CP and the evacuation near to PP part of the pond) which led to the movement of nutrients from CP to PP resulting in PP a better development of natural feed.

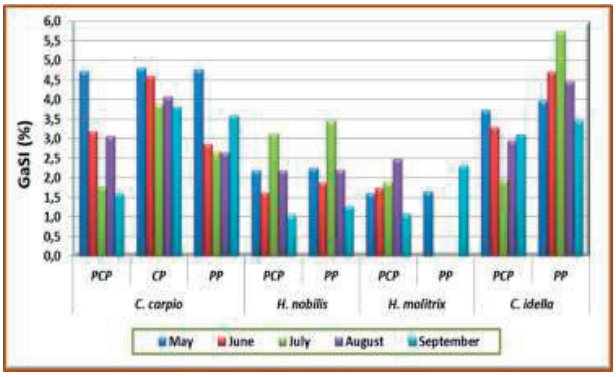

Fig. 12. Changes of the gastrosomatic index (GaSI) in response to different feeding regime

Although feed (mix of cereals) was administered in PCP, compared to PP pond, we can say that the fish were satisfied only with the food received and were no longer tempted to consume a large amount of natural feed. This aspect can explain the fact that the GaSI index was higher in case of Asian cyprinids in PP pond, because they had to eat only natural food, showing in the literature are presented that the consumption of only natural feed leads to gut increase in length and also in the weight than in the case of fish for which was administered feed (Koundal et al., 2013; Biswas, 1993).

At the same time, there was appeared the competition between carp species for the administered feed in PCP pond, for this reason the GaSI values were higher in the case of the common carp from CP pond.

Relative gut length (RGL)

Food and feeding habit of fish are important biological factors for selecting a group of fish for culture in ponds to avoid competition for food among themselves and live in association and to utilize all the available food (Dewan & Saha, 1979).

The relative gut length of the species may vary according to the difference in the food habits in different life stages (Biswas, 1993). The food preferences depend greatly on the nature of food available in the living habitat, environmental conditions, size or sexual stages of fish as well as inter and intra specific competition (Zacharia & Abdurahiman, 2004). Relative gut length (RGL) has been widely used to determine the feeding habits of fish such as herbivorous, carnivorous, omnivorous,

herbi-omnivorous or carni-omnivorous (Koundal et al., 2013, Dasgupta, 2002).
The food items of common carp is mostly omnivorous in nature. Among the food items zooplankton and debris and detritus were most dominant followed by the aquatic plant parts, phytoplankton, zooplankton, debris and detritus, insects and semi-digested food materials (Manon & Hossain, 2011). In our case fish is omnivorous because also feeds with zooplankton in culture ponds.

Regarding to the RGL the results are presented in Figure 13. A significant differences ($p<0.05$) between PCP and PP pond, at the level of all cyprinids, was registered in case of grass carp. The mean value of RGL obtained during the entire production cycle were 2.31±0.41 in CP, 2.04±0.61 in PP, respectively 2.11±0.43 in PCP pond for common carp, 6.29±1.51 in PP and 6.18±1.63 in PCP pond for bighead carp, 7.76±1.03 in PP and 7.64±1.18 in PCP pond for silver carp, 3.37±0.24 in PP and 2.89±0.32 in PCP pond for grass carp.

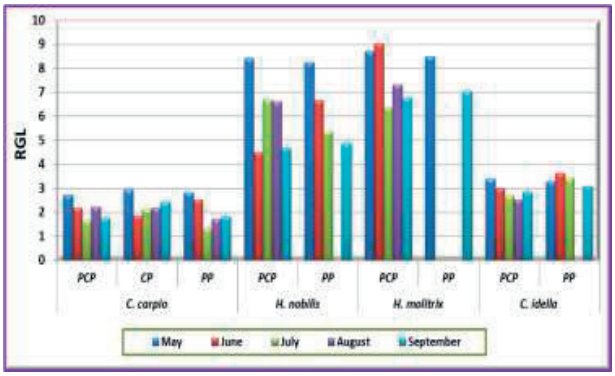

Figure 13. Changes of the relative gut length (RGL) in response to different feeding regime

Shafi et al. (2012) showed that the relative contribution of animal matter in the food of common carp clearly indicates that it is omnivorous in its feeding habit, which is also supported by its RLG which was present with a mean value of 1.87, value close to those obtained by us in case of common carp.
The analysis of gut content, Gastro-Somatic Index (GaSI) and Relative Length of the Gut (RLG) will definitely help in achieving basic information on overall biology of this fish species. Also, that organosomatic index is an appropriate bioindicator for endocrine disruption in fish consequent of chemical exposure (Dogan & Can, 2011).

CONCLUSIONS

Based on the obtained results of RGL, these cyprinids are considered as a omnivorous fish because the values are greater than 1.
Regarding to the GaSI value was observed low feeding intensity for cyprinids growth in polyculture carp pond (PCP) this is due to the emergence of food competition as opposed to those from the polyculture pond (PP part of CP-PP pond) who each had their own trophic niche as long as no additional food was administered.
In conclusion, this shows us that cyprinids can adapt to the natural feeding conditions in case of feed absence, a cereals mix in our case.
Also, these results of organosomatic indices can be considered useful as baseline data for further monitoring the fish condition, nutrition data and also inspirited the researchers to do further research in this area.

ACKNOWLEDGEMENTS

The work of Simionov Ira-Adeline was supported by the project "ANTREPRENORDOC", Contract no. 36355/23.05.2019, financed by The Human Capital Operational Programme 2014-2020 (POCU), Romania.
This work was supported by a grant of the Romanian National Authority for Scientific

Research and Innovation, CNCS/CCCDI – UEFISCDI, project PN-III-P2-2.1-PTE-2019-0697, within PNCDI III.
The authors are grateful for the technical support offered by the Grant POSCCE ID 1815, cod SMIS 48745 (www.moras.ugal.ro).

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

INDUCED SPAWNING AND EMBRYONIC DEVELOPMENT OF ORNAMENTAL CARP (*CYPRINUS CARPIO*) TROUGH THE APPLICATION OF PITUITARY EXTRACT

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Abstract

The present research aims to investigate the effects of using carp pituitary extract on spawning performance parameters of koi carp. The experiment was performed during the breeding season (may, 2020). The stimulation of koi carp sexual maturation was performed by injecting the breeders with carp pituitary suspension, at a water temperature of 22°C. A single injection with carp hypophysis, 3 mg/kg body weight (BW) for female and 2 mg/kg BW for male, was used for spawning induction, thus, resulting a successful ovulation. The eggs treatment was performed by applying 5.8 ml malachite green/100 liters of water. The following developmental stages of koi carp were observed and characterized: embryonic, hatchling, larval, post larval, fry and fingerling. The recorded prolificacy was 7460 eggs/kg female BW, considering that the koi carp female was at the 3rd deposit during the year 2020. The hatching started 72 hours after fertilization. Results of the current study indicate the successful induction of koi carp spawning by using carp pituitary extract.

Key words*: breeders, hatching, koi carp, pituitary extract, spawning.*

INTRODUCTION

Common carp (together with koi carp) is one of the most important freshwater fish species in aquaculture (Kucharczyk et al., 2008). The Japanese ornamental carp (*Cyprinus carpio* var. Koi) is famous for multifarious colors and patterns, making it commonly culture and trade across the world (Xue et al., 2018).

Fish reproduction has a considerable interest for both basic and applied research given the importance of this process for the maintenance of species, the high number of fish species (more than 25 000, representing a half of all living vertebrates), exhibiting very different reproductive tactics and strategies, their key position in vertebrate phylogeny and the increasing importance of aquacultured fish as a food resource (Servili et al., 2020).

Among the most significant advancements in the field of aquaculture during recent times is the development of techniques to induce reproduction in fish (Mudasir et al., 2014). Reproduction in fishes is regulated by external environmental factors that trigger internal mechanisms into action. The final event of the reproductive cycle, the release of eggs and sperm resulting in spawning, can be controlled by either placing the fish in an appropriate environment or by changing the fish's internal regulating factors with injected hormones or other substances.

One of the most important problems in modern aquaculture is obtained of high-quality gametes. For this purpose, several hormonal treatments are used to stimulate the maturity of gametes in the main marketable freshwater species (cyprinids, perches).

Production in recirculating systems also requires the use of directed reproduction techniques in order to increase the economic sustainability. In intensive aquaculture, the application of a stimulation method is necessary in order to assure high-quality gametes.

The application of a maturing agent usually influences not only the timing of maturation, but also the percentage of fish that spawn, as well as the quality and quantity of gametes.

Fishes show the broadest range of sexual patterns of any group of vertebrates (Godwin & Phillips, 2018). Hormonal stimulation in

common carp is a routine practice to enhance sperm production and control gamete maturation (Dietrich et al., 2019). Proper selection of breeders can be also considered as one of the keys to success in induced breeding. Thus, the breeders should be healthy, fully ripe and of medium size (Panda, 2016). Knowledge of fish reproductive biology is very important and contributes to the conservation of wild fish stocks through sustainable production technologies.

Therefore, understanding the reproductive aspects of fish is also very important for providing sound scientific advice in fishery management (Tessema et al., 2020). The aim of this study is to analyse the response of ornamental carp to injection with pituitary extract by evaluating the timing and duration of embryonic development stages, as well as the breeding performance in terms of prolificacy.

MATERIALS AND METHODS

The experiment was carried out during the spring of year 2020, at the Research Center for Modeling of recirculating aquaculture systems - Food Science and Engineering Faculty, "Dunărea de Jos" University of Galați.

Healthy, disease free, fully mature ripe fish exemplars, at the age 5 years, were selected as follows: the males with an average body weight (BW) of 445 g and the female with a BW of 372 g. Females and males were differentiated by shape: in females (♀), the body is plump and the genital opening is situated above the genital papilla. In males (♂), the body is slender and the genital opening is found behind the genital papilla (FAO, 2015).

On Thursday, May 7, year 2020, the breeders were brought from a pond rearing system (water temperature of 15ºC) and introduced, separately, in aquariums, at a water temperature of 20ºC. It must be pointed out that, during year 2020, until harvesting from pond, the water temperature was not favorable for koi carp reproduction.

Before the beginning of the experiment, fish were acclimatized during 2 weeks to the new stocking conditions. The breeders were fed with pellets at a feeding ratio of 2% BW, three times per day (8:00, 13:00, and 18:00). The oxygen level was over the concentration of 6 mg L^{-1}. The fish were subjected to a natural photoperiod, and the average temperature of the water was 22°C.

During the acclimatization the female spawn twice, without any stimulation (on May 9 and May 13). However, the feeding process continued, and the breeders were kept separately, as mentioned previously.

The injection of breeders with pituitary extract (prepared with saline solution and applied in a single dose) was proceed on May 19, year 2021, at 7 pm. The breeder's individual biomass was determined, and the doses were calculated as follows: 3 mg/kg BW for the female and 2 mg/kg BW for the male. The manipulation of the breeders was done with a damp cloth, without anaesthesia.

Pituitary extract was injected with sterile 1 mL syringes. The syringe needle was inserted into the base of the anal fin and the piston the pituitary extract was inserted into the general body cavity.

To avoid backflow of pituitary extract, the syringe needle was suddenly removed. After injection, breeders were placed in an aquarium with a protection grid on the bottom. To prevent infection of the eggs with *Saprolegnia*, a harmful factor specific to embryonic development, and considering that koi carp is an ornamental fish species, long-term treatment was performed with 5.8 mL of malachite green at 100 L of water.

The fertilized eggs of koi carp were observed as highly adhesive, demersal and spherical. Eggs samples were taken before fertilization and one time each hour, after the fertilization. Descriptions of the developmental stages were made by examining living specimens under the Novex Holland microscope with photo-camera.

RESULTS AND DISCUSSIONS

Determination of correct dosage of pituitary extract to be given to the breeders is very important and depends upon the size and state of maturity of the breeders as well as upon the state of maturity of the donor for the glands (Monjit & Chanda, 2014).

The time of injection depends upon the water temperature. In our case, at a temperature of 22°C, the injection was made 12 hours before the expected time for spawning.

The spawning period for common carp is typically at a water temperature ranging between 18-28°C, although spawning has been observed at water temperatures as low as 15°C (Tempero et al., 2006). Considering that ornamental carp, is a descendant of the common carp, it is assumed that the breeding conditions are similar.

According to Billard (1999), pituitary in ornamental carp is done in the same way as in common carp, but the percentage of ovulation in females is lower, often 50%, and fertility significantly lower (<100,000 eggs/kg body weight). In present experiment, the recorded prolificacy was 7460 eggs/kg female BW. Haniffa et al. (2007) performed a study in which spawning of koi carp was induced by intra-peritoneal injections of Ovaprim at a dose of 0.3 mL/kg body weight and the spawning was noticed 6 hours after the injection, at a temperature variation range of 26-28ºC. In the present study, the spawning was noticed 12hours after fertilization, at a water temperature of 22°C.

Changes in structure emphasize the thresholds between embryonic, larval and post-larval development from the onset of cleavage or epiboly, or at the time of organogenesis, respectively.

It has been observed that the eggs became translucent as development progressed. The diameter of the fertilized egg capsule ranged between 0.8-1 mm.

The incubation period of eggs depends largely on water quality parameters such as salinity and temperature (Kuo et al., 1973; Liao, 1975). Thus, in present study, after 72 hours of incubation, at a water temperature of 22ºC, the embryonic development ended and the hatching began, which lasted about 6-7 hours.

According to Kimmel et al. (1995), the newly fertilized eggs are found in the zygote period, until the appearance of the first division, at about 45 minutes from fertilization (Figure 1). The end of the zygote is marked by the appearance of the first division fold, which occurs near the animal pole. A view of the animal pole shows that the blastodisk has an ellipsoidal shape, as can be seen in Figure 1.

According to Stevens et al. (1998), embryonic development in carp is similar to that thoroughly studied by Kimmel et al. (1995) in zebrafish, *Danio rerio*.

After closing of zygote, the most important processes that take place during the blastula period are the entry of the embryo in the middle blastula period and the beginning of the epibolia. The period of the gastrula is characterized by a continuation of the epibolia. By the end of the gastrulation period, the yolk sac becomes completely swallowed.

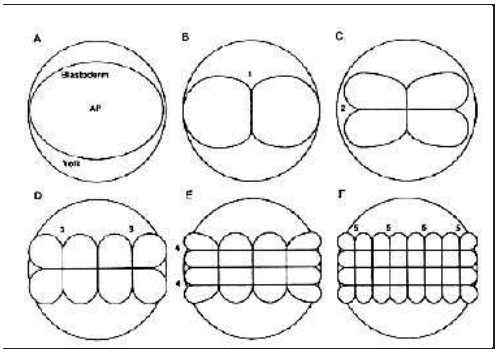

Figure 1. Graph of the animal pole during the first 5 divisions. The outer circle represents the vitellus, and the ellipsoidal ring in Fig. A represents the blastodisc before division. B-F shows the successive divisions, with the divisions seeming to cut the short axis of the blastodisc and the odd ones cutting the long axis (Kimmel et al., 1995)

In the present study, considering the description of Kimmel et al. (1995), the following stages were identified: segmentation, pharyngula and hatching period (Figure 2).

Epibolia closes because the blastoderm completely covers the yolk plug (100% epibolia) (6 hours after fertilization).

During the segmentation period the somites develop (10 hours after fertilization), the rudiments of the first organs become visible, the caudal buds become much more prominent and the embryo lengthens. The brain also acquires a tubular structure (16 hours after fertilization). The first morphologically differentiated cells and the first body movements appear (17 hours after fertilization). The somites appear consecutively on the trunk and tail (30 hours after fertilization). The rudiments of the eyes, the optical primordia (32 hours after fertilization), develop very early from the side walls of the diencephalon, giving the brain a arrowhead shape from the dorsal view (35 hours after fertilization). A characteristic strangulation that is important for

the 14-19 somite stage occurs in the posterior region of the yolk sac, where the tail buds end, and gives the vitellus a kidney shape (40 hours after fertilization). The rudiments of the crystalline and the otic ones appear digging the otic vesicle, which contains two very small otoliths at this stage (42 hours after fertilization).

This strangulation is quickly becoming more apparent. The strangled region develops into an elongation of the yolk sac, the extension of the yolk. The shape of the vitellus will continue to differ from the previous region, the vitellus ball (45 hours after fertilization). The caudal buds begin to project out of the embryo's body. The embryo enters the pharyngula period with a well-developed notochord and a recently completed set of somites extending to the end of the tail (50 hours after fertilization). In the hatching period, pigment cells differentiate; melanophores begin to differentiate at the beginning of this period, and the pigmentation looks much better during this period. The circulatory system is formed (60 hours after fertilization).

The heart starts beating right from the beginning of this period and has well-defined chamber formats. Blood begins to flow through a closed set of channels (63 hours after fertilization). During this period, the embryo continues to develop at about the same rate as in previous periods. The morphogenesis of many organs is now considerably complete and slowed down. The rapid development of the jaws, gills and pectoral fins can be observed. The development of pectoral fins is an important element especially for the beginning of the hatching period. Also specific to this period is the fact that the jaws develop, being visible even the very small mouth (70 hours after fertilization).

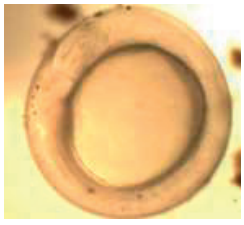

Figure 2.1. Gastrula

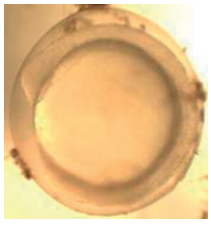

Figure 2.2. Segmentation (after 10 hours)

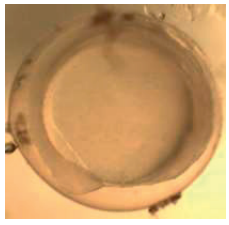

Figure 2.3. Segmentation (after 11 hours)

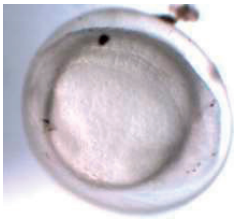

Figure 2.4. Segmentation (after 12 hours)

Figure 2.5. Segmentation (after 16 hours)

Figure 2.6. Segmentation (after 17 hours)

Figure 2.7. Segmentation (after 30 hours)

Figure 2.8. Segmentation (after 32 hours)

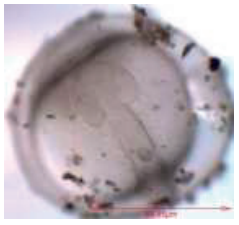

Figure 2.9. Segmentation (after 35 hours)

Figure 2.10. Segmentation (after 40 hours)

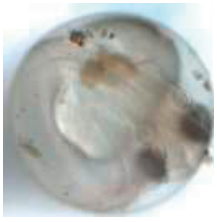

Figure 2.11. Segmentation (after 42 hours)

Figure 2.12. Segmentation (after 45 hours)

Figure 2.13. Segmentation (after 50 hours)

Figure 2.14. Hatching period (after 60 hours)

Figure 2.15. Hatching period (after 63 hours)

Figure 2. The development stages of ornamental carp

The length of larvae immediately after hatching was 6.3 mm, the mouth and anus were closed and the pectoral fin was formed (Figure 3).

Figure 3. The larva after hatch

The resorption of the yolk sac was observed 3 days after hatching (Figure 4). The larvae were fed egg yolk on the first day, *Artemia* nauplii the next day, then frozen rotifers.

Post larvae at 15 days after hatching had a total length of 12 mm, separate anal fin and dorsal membranes and fin ray. Since two month, the koi fry have been fed with frozen cyclops. On 06.08.2020, they had an average weight of 0.05 g/exp. Results of this study showed that successful induced spawning in ornamental carp was achieved by using a single dose of hypophysis, as described.

Figure 4. Larva after resorption of the yolk sac

CONCLUSIONS

The acclimatization of the female ornamental carp must be done with small variations in temperature in order not to spawn in the absence of the male.

Reproduction with pituitary extract can be used successfully in order to fructify the third deposit.

Further trials are now essential to standardize use of dosage and to gather additional information on the eggs and hatchlings of koi carp produced through pituitary extract treatment, such as their size, rate of growth, survival etc.

ACKNOWLEDGEMENTS

The work of Simionov Ira-Adeline was supported by the project "ANTREPRENORDOC", Contract no. 36355/23.05.2019, financed by The Human Capital Operational Programme 2014-2020 (POCU), Romania.
This work was supported by a grant of the Romanian National Authority for Scientific Research and Innovation, CNCS/CCCDI – UEFISCDI, project PN-III-P2-2.1-PTE-2019-0697, within PNCDI III.
The authors are grateful for the technical support offered by ReForm - MoRAS through the Grant POSCCE ID 1815, cod SMIS 48745 (www.moras.ugal.ro).

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

COMPARATIVE MORPHOMETRIC ANALYSIS OF MALE AND FEMALE HYBRIDS (F1 *ACIPENSER BAERII* × *ACIPENSER GUELDENSTAEDTII*) AT THE AGE OF SEVEN YEARS

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Abstract

A comparative analysis on 36 metric, 7 meristic features was performed and 7 morphometric indices were calculated in seven-year-old male and female hybrids (F1 Acipenser baerii x Acipenser gueldenstaedtii) grown under the same conditions on a super-intensive cage farm. The antiventral (P<0.05) and pecto-ventral (P<0.01) distance are bigger in female fish. Their head, compared to males, is wider, higher in the area above the eye and has a relatively larger space behind the eye (P<0.05). Female fish have a relatively larger eye diameter (P<0.01), wider mouth (P<0.001) and snout (P<0.001), higher dorsal (P<0.05) and anal, (P<0.05) and longer pectoral (P<0.001) and abdominal (P<0.05) fin. Male hybrids have a higher body relative to total length (P<0.01), a higher caudal stalk (P<0.05), and a longer anal fin (P<0.01). Their head has more massive and long snout (P<0.001), a bigger distance from the snout end to the mouth (P<0.001) and a greater width of the lower lip brake (P<0.05). The ratio of lower lip brake to the mouth length was higher (P<0.001) in male fish. The values of the high backed index (P<0.01) and the fatness index, including the body girth (P<0.05) in female fish are higher.

Key words: *aquaculture, exterior indices, hybridization, sturgeon.*

INTRODUCTION

Sturgeon aquaculture is developing at a very good pace (Bronzi et al., 2019). One of the most important aquaculture species are Siberian and Russian sturgeon. They are grown in pure form and are used as parental forms in hybridization. Sturgeons have a high ability to hybridize, and in the wild in sympatric populations hybridized species are often observed (Chebanov & Galich, 2013). Hybridization has been successfully applied in sturgeon aquaculture as a method to increase production efficiency (Miburo et al., 2018, etc.). Sturgeons have more than 20 interspecific hybrids (Havelka et al., 2011). Chebanov et al. (2018) emphasize that the cultivation of different Siberian sturgeon hybrids has practical significance for sturgeon aquaculture in different climatic and technological conditions.

Although phylogenetically Siberian and Russian sturgeons are closely related, the two species differ significantly; Russian sturgeon is a Ponto-Caspian anadromous species, while Siberian sturgeon is a potamodromous species inhabiting Siberian rivers and Lake Baikal (Ruban, 1997; Birstein & Ruban, 2004; Bogutskaya et al., 2013).

The qualities of hybrids between individual parental forms, when grown in aquaculture, have been the subject of a number of studies (Efimov, 2004; Filipova & Zuevsky, 2008; Linhartová et al., 2018; Shivaramu, 2019).

Morphometric analysis is used to characterize species and hybrids in sturgeon farming (Salmanov et al., 2016, etc.). It is an important part of creating test criteria for evaluating individual hybrids and breeds (Efimov & Krilova, 2006).

Morphometric studies are of paramount importance when working with fish farmed in aquaculture, as aquaculture conditions affect the morphotype of farmed fish (Shishanova & Kavtarov, 2015). Ruban (2019) points out that when breeding Siberian sturgeon in warm-water aquaculture there are major changes in a number of plastic and meristic features in the second generation.

Morev (1999) emphasizes that systematic morphometric analysis is an adequate tool for genetic study of collections of sturgeon and other fish species. The author points out that with the help of morphometric analysis the

structure of the artificial populations is clarified, reflecting the genetic heterogeneity of the latter in terms of their adaptation to certain growing conditions.

We set ourselves the goal to make a morphometric characteristic of male and female hybrids [F1 *Acipenser baerii* (Ab) x *Acipenser gueldenstaedtii* (Ag)] at the age of seven years, when grown in an industrial cage farm located in southeastern Bulgaria.

MATERIALS AND METHODS

The study was carried out with seven-year-old male (n = 25) and female (n = 25) hybrids (F1 Ab x Ag), from a net-cage farm, located in a warm water reservoir. According to its type, the reservoir refers to large and deep ones. Its area is 16.07 km^2, the volume is 532.9 x 10^6 m^3. The reservoir is located in South-East Bulgaria, at 41°37 'N latitude and 25°20' E longitude. It falls into the South Bulgarian climate zone, East Rhodope climate region. The average altitude is about 280 m. Fish of different age groups were grown in separate net-cages. The cages were 8 × 8 m in size, the water depth being 6 m. Each cage had a double polyamide net. Feeding was performed with commercial granulated sturgeon feed (Table 1).

Table 1. Composition of the commercial feed

Indices	Value	Indices	Value
Protein, %	46	Vitamin A, IU.kg^{-1}	10 000
Fat, %	15	Vitamin C, mg.kg^{-1}	520
Crude fibre, %	1.4	Vitamin E, mg.kg^{-1}	200
Ash, %	6.5	Vitamin D3, IU.kg^{-1}	2 303
Total P, %	1.03	Gross energy, MJ.kg^{-1}	21.0
Ca, %	1.4	Digestible energy, MJ.kg^{-1}	19.2
Na, %	0.3%		

Twenty five fish were randomly selected from the hybrid of different sex for morphometric analyzes at the end of the vegetation period (in November). The mean body weight of females was 5000.3±120 g and of males 4000.9±130 g.

Classical methods developed for the study of alive hydrobionts were applied for the study of sturgeon species (Pravdin, 1966; Krilova & Sokolov, 1981; Morev, 1999; Svirski & Skirin, 2005, etc.). In Table 2 the studied indicators and codes for their designation are presented. A measurement scheme proposed by Krilova & Sokolov (1981) specifically for sturgeon and their hybrids was used (Figure 1). Measurements of individual body parts are made with a caliper with an accuracy of 0.1 mm, a strip measure with an accuracy of 1 mm (for body girth measurements) and a graduated ichthyological board with an accuracy of 1 mm for measuring lengths, thicknesses and heights of the body.

Table 2. Metric and meristic features used in the study.

Features	Code
Total body weight, g	BW
Metric body features	
Total length, cm	TL
Fork length, cm	FL
Standart length, cm	SL
Antidorsal distance, cm	AD
Antiventral distance, cm	AV
Antianal distance, cm	AA
Maximum body width, cm	SC
Maximum body height, cm	H
Minimum body height, cm	H1
Tail stalk length - from the end of the anal fin to the roots of the middle rays of the caudal fin, cm	PL1
Tail stalk length - from the end of anal fin to the end of the middle rays of the caudal fin, cm	PL2
Dorsal fin length, cm	LD
Dorsal fin height, cm	HD
Anal fin length, cm	LA
Anal fin height, cm	HA
Pectoral fin length, cm	LP
Abdominal fin length, cm	LV
Pecto – ventral distance, cm	PV
Ventro – anal distance, cm	VA
Maximum body girth, cm	CC
Metric head features	
Head lenght, cm	C
Snout length, cm	R
Maximum head height (before the 1st dorsal bony scute), cm	HC
Minimum head height (above the eye), cm	HCO
Behind eye area length, cm	CP
Horizontal eye diameter, cm	O
Inter orbital distance, cm	IO
Maximum head width, cm	BC
Distance from the beginning of the snout to a line passing through the middle of the front barbels` roots, cm	RC
Distance from the end of the snout to the mouth cartilaginous arch, cm	RR
Distance from the middle barbels` roots to the mouth cartilaginous arch, cm	RL
Longest / lateral / barbel`s length, cm	LC
Snout width at the middle barbels` roots, cm	SRC
Snout width at the mouth cartilaginous arch, cm	SRR
Mouth width, cm	SO
Lower lip`s break width, cm	IL
Meristic features	
Number of dorsal bony scutes	SD
Number of lateral bony scutes from the left side of the fish	SL1
Number of lateral bony scutes from the right side of the fish	SL2
Number of ventral bony scutes from the left side of the fish	SV1
Number of ventral bony scutes from the right side of the fish	SV2
Number of rays in the dorsal fin	D
Number of rays in the anal fin	A

Morphometric indices were calculated on the basis of morphometric measurements (Table 3). For statistical data processing IBM SPSS Statistics 21 was used.

RESULTS AND DISCUSSIONS

Metric features of the body in seven-year-old Siberian and Russian sturgeon hybrid of different sexes are shown in Table 4. Except the two indicators characterizing body height

(H, H1) and anal fin length (LA), female fish had higher average values. The total length of the body in females varied from 93.60 to 109 cm, and in males - from 91.8 to 107.00 cm. There is a slight variation (<10%) of the features related to the proportions of the individual body parts. The variation is higher (11.10%) only along the abdominal fin of female individuals.

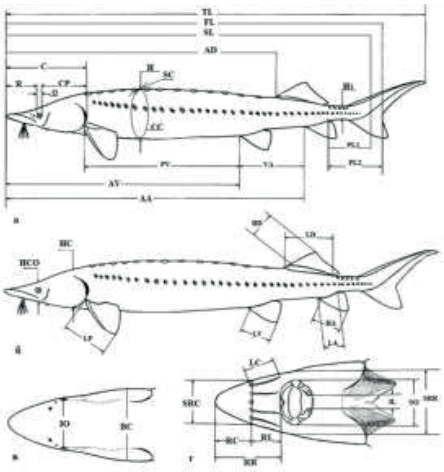

Figure 1. Sturgeon fish measurements scheme (Krylova and Sokolov, 1981; Svirski and Skirin, 2005)

Table 3. Morphometric indices

Indices	
CFF	Fulton's coefficient [(BW/SL^3)*100], %
IC	Condition index [BW/(SL*H*CC)*100], %
ICR	Modified Fulton's coefficient by Jones et al., 1999 (acording Richter et al., 2000) [BW/(SL^{2}H)*100]
IHB	High-backed index (SL/H)
IBB	Broad-backed index [(SC/SL)*100], %
ILH	Long-headed index [(C/SL)*100], %
IH	Hardness index [(CC/SL)*100)], %

Generally, in sturgeon species, sexual dimorphism is poorly developed, but some morphometric characteristics can show differences between the two sexes. In a study of *Huso huso* Falahatkar & Poursaeid (2014) did not establish a reliable relationship with sex in most of the studied morphological indices, but found one at the ratio of fork length to distance of snout to anterior of dorsal fin.

Maltsev & Merkulov (2006) developed a method for biometric sex determination in sturgeons based on head measurements using discriminant analysis. Barulin (2018) has developed a sex determination system in *A. ruthenus* based on the morphological features of dorsal scutes. Podushka (2008) reported differences in the shape of the pectoral fins in the Amur sturgeon *A. schrenckii*. Morphometric characteristics depend on the hybridization scheme and the participation of one species as the maternal or paternal form in interspecific hybrids. In case of hybridization between Russian and Siberian sturgeon more often research concerns the GUBA hybrid (with maternal form Russian sturgeon). The hybrid was first obtained in 1979 in Russia at the VNIRO Research Institute, and studies show that GUBA have better productive performance than their parental forms (Filipova & Zuevsky, 2008).

Hybrids, in aquaculture conditions, have higher survival and growth compared to purebred parental forms (Shivaramu et al., 2019). Efimov (2004) in a study of the GUBA hybrid found that most metric features are inherited patroclinically (by father), with the number of ventral and dorsal scutes also having patroclinal and lateral matroclinal (by mother) inheritance. The author notes that the variability in the hybrid is less compared to the parental forms on meristic features.

The hybrid with the participation of Siberian sturgeon as a maternal form (Ab x Ag) is less popular in sturgeon farming, but is assessed as promising for commercial sturgeon aquaculture (Chebanov et al., 2018). It shows good results in cultivation in various production technologies (Iskhakova & Khulmanova, 2014; Nikolova & Bonev, 2020). Such a hybrid in aquaculture conditions at the age of 5 years forms normally developed gonads, similar to pure Siberian sturgeon of the same age (Linhartová et al., 2018).

Szczepkowski et al. (2002) noted the importance of developing appropriate criteria, including meristic features - bony scutes, finrays and some metric features, to identify different Sturgeon hybrids.

Usually the ratio of individual measurements to the body length of the fish is calculated in morphometric studies. The analysis of the ratio of individual measurements to the body total length obtained in our study showed a significant difference on several indicators between individuals of different sexes (Table 5).

H/TL values (P<0.01) were higher in males than in females; H1/TL (P<0.05); LA/TL (P<0.01) and lower in AD/TL (P<0.05); AV/TL (P<0.05); AA/TL (P<0.05); HD/TL (P<0.05); HA/TL (P<0.05); LP/TL (P<0.001); LV/TL (P<0.05); PV/TL (P<0.01).

Table 4. Metric features of the body, cm

Features	Sex	X	Min	Max	±Sx	CV
TL	F	102.00	93.60	109.00	0.85	4.06
	M	99.3	91.8	107.00	0.86	4.25
FL	F	89.20	82.00	96.50	0.69	3.81
	M	86.00	75.3	93.3	0.85	4.82
SL	F	83.60	76.10	89.40	0.65	3.79
	M	80.5	73.00	87.60	0.70	4.27
AD	F	63.80	60.20	67.40	0.51	3.94
	M	60.9	54.60	67.00	0.54	4.37
AV	F	55.30	49.30	76.60	1.04	9.22
	M	51.60	46.90	57.00	0.54	5.13
AA	F	69.30	64.60	73.30	0.58	4.07
	M	66.10	58.80	72.70	0.68	5.07
SC	F	10.60	9.20	11.70	0.11	4.98
	M	10.20	9.00	11.40	0.11	5.43
H	F	11.90	11.00	12.90	0.12	4.74
	M	12.20	10.60	13.60	0.13	5.40
H1	F	3.30	2.94	3.62	0.03	5.19
	M	3.35	2.92	3.66	0.04	6.12
PL1	F	8.83	7.90	10.10	0.11	6.28
	M	8.61	7.85	10.60	0.15	8.30
PL2	F	14.80	13.10	16.30	0.16	5.25
	M	14.20	12.90	15.40	0.15	5.03
LD	F	11.50	9.10	12.70	0.16	6.77
	M	10.9	9.60	12.50	0.16	7.03
HD	F	9.84	8.50	11.30	0.14	7.07
	M	9.13	8.26	10.50	0.11	6.16
LA	F	5.55	4.00	6.30	0.10	9.16
	M	5.85	4.68	6.70	0.10	8.39
HA	F	10.20	7.80	11.70	0.18	8.64
	M	9.56	8.55	10.70	0.13	6.86
LP	F	13.2	11.70	14.70	0.16	6.07
	M	11.50	9.95	13.40	0.18	7.49
LV	F	8.48	5.39	9.80	0.19	11.10
	M	7.87	6.63	9.05	0.12	7.39
PV	F	35.60	31.80	38.30	0.34	4.66
	M	33.50	29.40	38.00	0.43	6.28
VA	F	15.20	13.70	17.30	0.18	5.89
	M	14.90	12.70	17.30	0.20	6.73
CC	F	37.40	36.00	40.40	0.33	4.26
	M	36.30	33.40	40.40	0.39	5.32

The differences between individuals of different sexes are clearly seen in Figure 2, where the exterior body and head profiles are presented.

A shorter head, broader body and longer back are desirable for sturgeon hybrids (Szczepkowski et al., 2002). Body shapes are associated with meat-producing characteristics. Szczepkowska et al. (2011) found the best commercially advantageous body proportions in five-year-old fish, with a relative head length of 24% of TL and a body height of 11.82% of TL studying the characteristics of the Siberian and Green Sturgeon hybrid.

The hybrids in our study are seven years old, which suggests a well-formed morphotype. The relative length of the head from TL is smaller than in the above study and is 18.4% in female and 18.6% in male fish, while in body height (H) the differences are not so great (in females 11.60%, and in males 12.30%).

Table 5. Individual measurements to the total length ratio of seven year old hybrid (Ab x Ag) body, %

Features	Sex	X	Min	Max	±Sx	CV
FL/TL	F	87.20	85.30	87.90	0.20	1.11
	M	86.60	75.10	89.80	0.58	3.31
SL/TL	F	81.70	78.80	84.20	0.27	1.60
	M	81.10	70.90	83.80	0.54	3.24
AD/TL	F	62.40*	60.00	64.60	0.27	2.10
	M	61.30*	58.40	63.90	0.30	2.37
AV/TL	F	54.00*	49.30	70.40	0.80	7.26
	M	51.90*	49.40	54.20	0.26	2.44
AA/TL	F	67.70*	63.90	70.10	0.29	2.09
	M	66.50*	60.60	69.90	0.41	3.01
SC/TL	F	10.30	9.08	11.00	0.10	4.71
	M	10.30	9.10	11.10	0.11	5.08
H/TL	F	11.60**	11.00	13.20	0.11	4.47
	M	12.30**	10.70	14.10	0.16	6.37
H1/TL	F	3.23*	2.91	3.85	0.04	5.75
	M	3.37*	2.91	3.83	0.04	6.35
C/TL	F	18.40	16.40	20.10	0.20	5.25
	M	18.60	16.50	21.0	0.18	4.83
PL1/TL	F	8.64	7.64	10.10	0.13	7.09
	M	8.68	7.48	10.80	0.14	7.94
PL2/TL	F	14.40	12.80	16.60	0.18	5.98
	M	14.30	13.00	15.20	0.13	4.39
LD/TL	F	11.24	9.72	12.62	0.12	5.26
	M	11.02	9.71	12.18	0.14	6.01
HD/TL	F	9.63*	8.02	10.70	0.13	6.77
	M	9.20*	8.22	10.60	0.13	6.76
LA/TL	F	5.43**	4.27	6.02	0.09	7.98
	M	5.89**	4.73	6.80	0.10	8.41
HA/TL	F	10.00*	8.21	11.10	0.14	6.96
	M	9.63*	7.99	10.40	0.12	6.20
LP/TL	F	12.90***	11.10	14.60	0.19	7.17
	M	11.60***	9.93	13.10	0.18	7.61
LV/TL	F	8.29*	5.09	9.71	0.18	10.50
	M	7.93*	6.99	8.83	0.10	5.89
PV/TL	F	34.80**	31.40	36.10	0.25	3.52
	M	33.70**	31.80	36.00	0.29	4.15
VA/TL	F	14.90	13.80	16.00	0.11	3.69
	M	15.00	13.40	17.10	0.15	4.89
CC/TL	F	36.60	34.10	40.60	0.32	4.35
	M	36.50	32.90	40.00	0.34	4.56

Differences between the values within the feature are significant: ****P<0.001, **P<0.01, *P<0.05*

Chebanov et al. (2018) indicate that the hybrid of Russian and Siberian sturgeon is more similar to Siberian, and in most metric and meristic features the hybrid occupies an intermediate position with a bias to the paternal species. Efimov (2004) found that age variability of morphometric features is observed in the Russian and Siberian sturgeon hybrid. The author notes that at an older age the hybrid phenotypically begins to resemble the Russian sturgeon more than at an younger age, and with age the head proportions change (the relative head length to the body length decreases and the snout length to the head length too). The results of the head metric features study in the hybrid of both sexes are presented in Table 6.

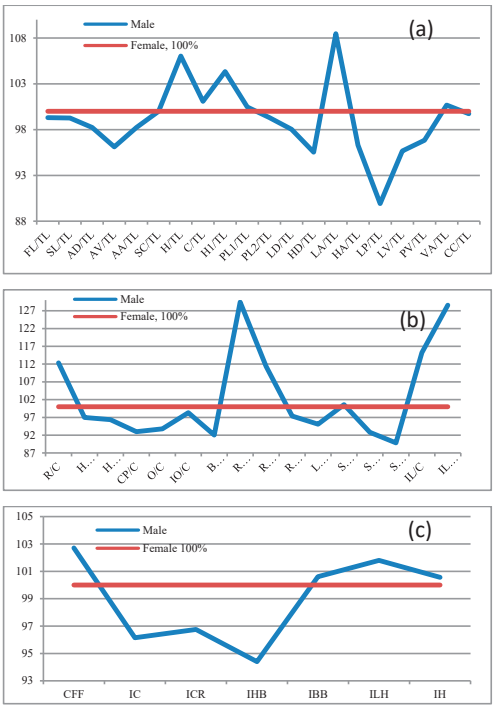

Figure 2. Exterior body profiles (a), head (b) and indices (c) in the hybrid of different sex.

Table 6. Metric features of hybrid (Ab x Ag) head at the age of seven years, cm

Features	Sex	X	Min	Max	±Sx	CV
C	F	18.8	17.30	21.00	0.20	5.25
	M	18.50	16.30	19.70	0.15	3.94
R	F	7.03	5.97	7.94	0.09	6.16
	M	7.75	6.30	8.95	0.13	8.01
HC	F	8.42	7.50	9.25	0.09	5.35
	M	8.02	7.12	8.88	0.09	5.80
HCO	F	5.76	5.20	6.30	0.07	5.94
	M	5.44	4.80	6.00	0.07	6.16
CP	F	10.8	9.45	11.60	0.12	5.35
	M	9.80	8.60	10.70	0.11	5.29
O	F	1.46	1.32	1.70	0.02	6.00
	M	1.34	1.21	1.45	0.02	6.23
IO	F	6.44	5.10	6.94	0.08	5.81
	M	6.20	5.70	6.70	0.06	4.44
BC	F	8.46	7.45	9.44	0.09	5.11
	M	7.63	6.92	8.25	0.07	4.57
RC	F	3.02	2.41	3.60	0.06	9.58
	M	3.83	2.45	4.80	0.13	16.10
RR	F	7.32	6.50	8.00	0.09	5.72
	M	8.00	6.05	9.28	0.14	8.56
RL	F	4.45	3.60	5.10	0.06	6.89
	M	4.25	3.70	4.82	0.06	6.94
LC	F	3.94	2.80	4.80	0.09	10.60
	M	3.68	2.50	4.30	0.10	13.10
SRC	F	5.87	4.60	6.77	0.08	6.79
	M	5.78	5.00	6.42	0.08	6.90
SRR	F	8.84	8.35	9.78	0.09	4.94
	M	8.04	7.30	8.78	0.08	4.73
SO	F	6.75	6.20	7.48	0.08	5.71
	M	5.94	5.20	6.70	0.07	5.70
IL	F	1.05	0.40	1.50	0.05	24.50
	M	1.18	0.80	1.75	0.05	22.60

The variation is low (<10%) for most of the features.

Mean levels of variation (16.10%) were found with respect to head length from the snout to the roots of the anterior barbels in male fish; the length of the lateral barbels in both sexes (10.60 and 13.10%). Significant variation (22.60 and 24.50%) in both sexes was found in the width of the lower lip break.

Table 7 shows the relationships between the metric features of the head and its length. The shape and proportions of individual parts of the head in sturgeons are an important diagnostic features.

Table 7. Head metric features to head length ratio of seven years old hybrid (Ab x Ag), %

Features	Sex	X	Min	Max	±Sx	CV
% of head length						
R/C	F	37.30***	34.30	39.50	0.27	3.61
	M	42.00***	36.60	46.50	0.51	5.97
HC/C	F	44.80	39.00	53.60	0.72	7.89
	M	43.50	37.70	49.10	0.52	5.84
HCO/C	F	30.60*	26.60	33.70	0.36	5.83
	M	29.50*	26.50	32.50	0.38	6.32
CP/C	F	57.20***	51.70	60.10	0.38	3.27
	M	53.20***	47.80	58.30	0.57	5.25
O/C	F	7.74**	7.10	8.63	0.09	6.00
	M	7.26**	6.60	8.77	0.10	6.84
IO/C	F	34.20	30.80	38.3	0.33	4.80
	M	33.60	29.90	35.90	0.33	4.83
BC/C	F	45.00***	39.50	48.70	0.55	6.00
	M	41.40***	38.10	46.30	0.38	4.50
RC/C	F	16.00***	13.70	17.90	0.22	6.73
	M	20.70***	15.00	26.50	0.64	15.10
RR/C	F	38.90***	35.60	41.70	0.29	3.60
	M	43.30***	37.10	48.20	0.64	7.24
RL/C	F	23.60	20.90	25.30	0.23	4.83
	M	23.00	20.80	26.40	0.32	6.73
LC/C	F	21.00	16.20	25.90	0.50	11.60
	M	19.90	13.30	23.20	0.49	12.00
SRC/C	F	31.20	27.60	35.20	0.39	6.11
	M	31.30	27.10	35.20	0.40	6.23
SRR/C	F	47.00***	42.40	50.30	0.44	4.61
	M	43.60***	38.30	48.30	0.47	5.23
SO/C	F	35.80***	33.20	38.10	0.26	3.53
	M	32.20***	29.40	36.90	0.39	5.90
IL/C	F	5.58*	2.13	8.13	0.28	24.70
	M	6.43*	4.11	9.64	0.31	23.50
% of the mouth width						
IL/SO	F	15.50***	9.54	22.10	0.75	23.70
	M	20.00***	14.00	28.70	0.91	22.40

Differences between the values within the feature are significant: ***P<0.001, **P<0.01, *P<0.05

The snout in male fish occupies a larger share of the head (P <0.001) than in females. The snout of female fish occupies from 34.3 to 39.50% of the head length, and in males from 36.60 to 46.50%, respectively.

There is no significant difference between female and male hybrids in the ratio of the maximum height of the head to its length. A significant difference in favor of female fish (P<0.05) was found in relation to the minimum height of head to its length.

The ratio behind eye area length (P <0.001); eye diameter (P<0.01); the head width

(P<0.001), the mouth (P<0.001) and the snout at the cartilaginous arch (P<0.001) to the head length were significantly higher in female fish; and in males, respectively, the length from the beginning of the snout to the roots of the barbels (P<0.001); from the end of the snout to the mouth arch (P<0.001); the width of the lower lip break (P<0.05) (Table 7; Figure 2). The ratio of the lower lip break to the mouth length in male fish is also significantly higher (P<0.001).

The morphometric indices of the hybrid are shown in Table 8.

Table. 8. Morphometric indices in a seven-year-old hybrid (Ab x Ag)

Indices	Sex	X	Min	Max	±Sx	CV
CFF	F	0.91	0.84	1.13	0.01	7.90
	M	0.93	0.81	1.33	0.02	12.50
IC	F	14.30*	12.50	15.60	0.14	4.65
	M	13.70*	11.60	15.90	0.23	8.08
ICR	F	6.39	5.43	7.06	0.08	6.00
	M	6.18	5.00	8.12	0.13	10.30
IHB	F	7.03**	6.14	7.40	0.06	4.02
	M	6.64**	11.30	7.73	0.09	6.60
IBB	F	12.50	11.90	13.70	0.11	4.40
	M	12.70	11.30	13.90	0.15	5.63
ILH	F	22.50	20.10	24.40	0.18	4.02
	M	22.90	20.60	25.60	0.20	4.31
IH	F	44.80	41.70	49.90	0.39	4.23
	M	45.10	40.20	51.10	0.48	5.24

Differences between the values within the feature are significant: ***P<0.001, **P<0.01, *P<0.05

Exterior indices are taken as a basis for conducting selection work with fish. High-backed and hardness index are especially important. They characterize producers and are directly related to productivity indicators. Khabzhokov et al. (2018) found that the hardness index reflects very well the characteristics of each individual in body weight, length, height, body thickness, gonadal development and obesity in selection work with carp. We did not find significant differences in CFF and ICR fatness indices between female and male fish but in female fish the IC values were significantly higher (P<0.05). The IC index shows the girth of the body, with the more voluminous abdominal area usually associated with more massive gonads in females. The values of the high-backed index (P<0.01) are higher in female fish (Figure 2). According to the other indices, the differences between the two sexes are not significant. The variability of features in fish is also related to the fish age and the rearing conditions. A great phenotypic variability is characteristic of Siberian sturgeon (Ruban, 2019). In our study of the hybrid, most of the calculated indices varied low (<10%). Higher, to average variation values were found in the CFF and ICR index for male fish. Both indices are related to fish fattening. The analysis results of the meristic features in the hybrids of different sex are presented in Table 9.

Table. 9. Meristic features of seven-year-old hybrid (Ab x Ag)

Features	Sex	X	Min	Max	±Sx	CV
SD	F	13.10***	10	16	0.26	9.67
	M	11.70***	9	14	0.23	9.70
SL1	F	35.40*	29	41	0.62	8.58
	M	33.40*	27	40	0.68	10.00
SL2	F	35.2*	27	39	0.52	7.20
	M	33.60*	27	39	0.72	10.50
SV1	F	9.88	8	8	0.23	11.40
	M	9.60	7	13	0.26	13.40
SV2	F	9.96**	8	12	0.23	11.40
	M	9.32**	7	11	0.23	12.30
D	F	38.40*	28	45	0.75	9.60
	M	36.10*	31	44	0.68	9.19
A	F	21.00*	13	26	0.70	16.30
	M	18.90*	15	24	0.45	11.80

Differences between the values within the feature are significant: ***P<0.001, **P<0.01, *P<0.05

Meristic features are important in sturgeon taxonomy (Sergeev, 2020). The author finds in his research for a Russian sturgeon SD 12.5 (10-17); SL 35.03 (26-48); SV 9.6 (7-12).

Romanov & Skirin (2011) found a high level of morphological variability in the number of bony scutes in a study of different sturgeon species and hybrids meristic parameters, with a particularly large amplitude found in the lateral ones. In the study of the authors of a complex hybrid of Siberian and Russian sturgeon (Ag x Ab) x (Ag x Ab) the number of bone shields was as follows - SL 40.46 ± 0.33 (33-47); SV 9.4 ± 0.08 (7-12); SD 13.46 ± 0.17 (11-16).

Szczepkowska et al. (2011) in the hybrid of Siberian and Green sturgeon found meristic features as follows: SD 9.16 ± 0.82; SL 33.05 ± 2.09; SV 8.69 ± 0.86; D 35.67 ± 2.59; A 22.92 ± 1.91, the established values for the number of bony scutes were less than the Siberian sturgeon, and the number of rays of D and A were almost the same. The authors found that there is an age variability on meristic features in this hybrid.

Most morphometric studies do not indicate the sex of the tested fish. There is no significant difference between fish of different sexes of the ventral scutes number on the left side in our study, and on the right side female fish have a

larger number (P≤0.01), and their number varies from 8 to 12. The number of dorsal bony scutes in female fish is larger (P≤0.001), ranging from 10 to 16. The indicator varies from 9 to 14 in male fish. The difference in favor of female fish and the number of lateral scutes on the left and right is significant (P<0.05).

Female fish have a significantly higher (P≤0.05) number of rays in the dorsal and anal fin. The analysis generally shows a higher variation in the number of anal fin rays and in the number of ventral shields. The obtained results in our study on the characteristics of morphometric features of the Siberian and Russian sturgeon hybrid can be useful not only for aquaculture, but also when working with natural populations. The issues of "genetic pollution" are especially relevant for sturgeons in the wild. Development of sturgeon farming is one of the tools aimed at reducing the anthropogenic pressure on endangered natural populations, at the same time sturgeon aquaculture carries potential risks. Chebanov & Galich (2013) emphasize that a damage to fragile natural sturgeon populations can occur in case of fish introduction from aquaculture farms into the environment. Friedrich (2018) notes that the ability of sturgeons to hybridize and to produce fertile offspring is one of the threats to natural populations when non-native sturgeon species enters their range. Cases of natural reproduction of Siberian sturgeon have already been reported in the Danube, as well as the presence of hybrid forms between it and the local Sterlet (Ludwig et al., 2009). In connection with the above, it is important to have databases on the morphometric characteristics of different interspecific sturgeon hybrids.

CONCLUSIONS

The comparative characteristic of female and male individuals of a Siberian and Russian sturgeon hybrid (F1 Ab x Ag) at the age of seven show that there are differences in morphometric characteristics between the two sexes. Female hybrids have a bigger high backed index and have a higher degree of fatness, expressed by the IC index. In female fish, the antiventral and pectoventral distance is greater. The head of the female hybrids, compared to the male, is wider, higher in the area above the eye and has a relatively larger space behind the eye. Female fish have a relatively larger eye diameter; relatively wider mouth and wider snout at the cartilaginous arch; higher dorsal and anal fin and longer pectoral and abdominal one. Male hybrids have a higher body relative to the absolute length of the body, and a higher caudal stalk. Their anal fin is longer. The head of male fish compared to females has a more massive and longer snout; greater distance from the snout end end to the mouth and greater width of the lower lip break. The ratio of the mouth break to its length of male fish is higher.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

LABORATORY TESTING OF THE AMERICAN BLUE CRAB'S (*CALLINECTES SAPIDUS* RATHBUN, 1896) CAPACITY OF ADAPTATION TO AQUACULTURE SYSTEMS AT THE ROMANIAN COAST

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Abstract

The blue crab, Callinectes sapidus (Rathbun, 1896), is native to the Western Atlantic, supporting extensive fisheries and more recently aquaculture pursuits. It has become established as a non-native species in the Mediterranean, while in the Black Sea it was first reported decades ago, near the Bulgarian coast. A first specimen was collected at the Romanian coast in 1998 and, since then, individuals of this species have been reported several times. Due to its high market value and potential for aquaculture, we investigated the adaptation of the blue crab to captivity conditions. One adult was caught in fishing nets in the Mamaia Bay and transported to NIMRD's aquaculture laboratory. The crab was sexed (male) and measured (carapace width = 205 mm; carapace length = 100 mm; biomass = 537.58 g), and subsequently placed in a small PAS (pump-ashore system). Live mussels were introduced in the tank and the C. sapidus specimen was immediately observed feeding actively with the mollusks. Additionally, small fish were offered, being rapidly consumed. The overall observed behavior in captivity encourages aquaculture endeavors for this valuable species.

Key words*: aquaculture, Black Sea, captivity, crustaceans, pump-ashore system (PAS).*

INTRODUCTION

The American blue crab, *Callinectes sapidus* Rathbun, 1896, is of major interest to fisheries in the tropical and subtropical waters of the Western Atlantic. It supports large valuable commercial and recreational fisheries in the temperate areas of the Atlantic and Gulf coasts of the United States. It is the most widely harvested and consumed crab in the US, which is also the world's main producer of blue crab (Millikin & Williams, 1984). The Chesapeake Bay has supported an abundant blue crab population with an intense fishery, which currently supplies over one-third of all US commercial blue crab landings (Miller et al., 2011).

C. sapidus is a decapod crustacean, belonging to the family Portunidae. The carapace is more than twice as broad as long; it has nine blunt to acuminate teeth (outer orbital tooth and strong lateral spine included) on arched anterolateral margin. The pincers are strong, dissimilar and ridged longitudinally; the fifth legs are flattened in the form of paddles. The colour is greyish, bluish, or brownish green of varying shades and tints are present dorsally on carapace and chelipeds (Tavares, 2002).

Widely tolerant of salinity and temperature limits, the American blue crab is found in shallow waters near the coasts, especially at the mouths of rivers and streams, on sedimentary, sandy or muddy bottoms (Skolka & Gomoiu, 2004). Metabolic activity is slowed at temperatures below 20°C and blue crabs tend to become less active. When air temperatures drop below 10°C, adult crabs leave shallow, inshore waters and seek deeper areas where they bury themselves and remain in a state of torpor throughout the winter (Rome et al., 2005).

C. sapidus is native to the Western Atlantic (Chesapeake Bay), from Nova Scotia, Maine and northern Massachusetts to Argentina, including Bermuda and the Antilles (Tavares, 2002). It has also been successfully introduced, accidentally or intentionally, into both Asia and Europe. Accidental introductions have been attributed to larval transport via ship ballast water (Skolka & Gomoiu, 2004). It was introduced in Europe (Denmark, Netherlands, and adjacent North Sea, France, Golfo di Genoa); northern Adriatic; Aegean, western Black and eastern Mediterranean Seas. It has also been introduced to Japan. It is now rather

abundant in parts of the northern and eastern Mediterranean Sea and Japan (Skolka & Gomoiu, 2004).

The blue crab has become established as a non-native species in the Mediterranean basin (Holthuis, 1961). The blue crab was first recorded in the Mediterranean in Egyptian waters in the 1940s (Banoub, 1963). Subsequently, it has been reported in coastal waters off Italy (Giordani-Soika, 1951, as *Neptunus pelagicus*), Israel (Holthuis & Gottlieb, 1955), Greece (Kinzelbach, 1965) and Turkey (Kocataş & Katağan, 1983). Most recently, it has been reported in the Bay of Biscay, along the northwestern coast of Spain (Cabal et al., 2006) and the Sacca di Goro lagoon, an area located in the northern part of Italy (Manfrin et al., 2015).

In the Black Sea it was reported decades ago, near the Bulgarian coast (1968), as isolated specimens (Müller, 1986). To date, there are approximately fifteen records of occurrences of *C. sapidus* in the Black Sea (including brackish-water areas such as the Dnieper-Bug estuary) and the Sea of Azov (Snigirev et al., 2020). The first-time findings of *C. sapidus* on the north-western Black Sea shelf are in line with earlier assumptions on the hydrological characteristics of this area: relatively low salinity (compared to the Mediterranean) and a muddy substrate, an appropriate environment for *C. sapidus* proliferation (Snigirev et al., 2020). An increased number of *C. sapidus* findings in the Black Sea region during the last decade suggests the species to be naturalised in the area and widespread in the coastal waters of the Black Sea (Tokarev & Shulman, 2007).

A first specimen was collected at the Romanian coast in the summer of 1998 - a large male (85 mm long shell, 196 mm wide between the two lateral spines), and, a year later, a female would be collected in the same area - southern Romanian coast (Skolka & Gomoiu, 2004). Subsequently, specimens of this species have been reported several times in the southern part of the coast (Micu & Abaza, 2004; Nicolaev et al., 2004); one of them, captured alive, was kept in captivity for several months at the Aquarium in Constanţa. In all probability, in the southern part of the Romanian coast there is already a population of American blue crab, but its numbers are small (Skolka & Gomoiu, 2004). In recent years, isolated individuals have been reported (in 2016), accidentally caught by fishermen in the southern part of the littoral (*verbal information*). The latest records of *C. sapidus* were reported in 2020: one individual was caught in the Mamaia Bay (September 2020), one in Agigea (October 2020) and another one in Costinesti (November 2020). Production of soft-shell blue crabs represents one of the oldest aquaculture industries in the United States. The industry is dependent upon the capture of pre-molt (peeler) crabs from the wild fishery which are held in shedding trays until they molt (Oesterling, 1995). Commercial exploitation of the blue crab is rapidly increasing worldwide. One possible way to overcome the dependence on natural stocks for soft-shell crab industry is to rely on the development of technologies for reproduction, larval rearing, and cultivation of crabs in captivity (Zohar et al., 2008).

In this context, the purpose of this research was to investigate the species' capacity of adaptation to aquaculture systems at the Romanian coast.

MATERIALS AND METHODS

On 9 September 2020, one *C. sapidus* individual was accidentally caught by fishermen in the Mamaia Bay (trap net) and subsequently transferred to NIMRD's aquaculture laboratory (Figure 1).

Figure 1. *C. sapidus* specimen caught in fishing nets in the Mamaia Bay (Original photos)

After accurate identification of the species, the crab was sexed, based on the shape of the abdomen, as an adult male (Figure 2).

Figure 2. Sex determination of the *C. sapidus* specimen (adult male) (Original photo)

Initial biometric measurements of the individual were performed: carapace width (CW) = 205 mm, carapace length (CL) = 100 mm (Figure 3) and biomass = 537.58 g (Figure 4).

Figure 3. Carapace width and length measurements of the *C. sapidus* individual (Original photos)

Figure 4. Biomass of the *C. sapidus* individual (Original photo)

After one week of acclimation in a fiberglass tank, during which the animal refused to feed (Figure 5 up), it was transferred to a small pump-ashore system (PAS), provided with a natural substrate (rocks and sand) (Figure 5 down).

Figure 5. *C. sapidus* during the first week of acclimation (up) and after transfer to the PAS tank (down) (Original photos)

Land-based pump-ashore systems (PAS) are a type of flow-through system constructed on land adjacent to natural water bodies from which water is diverted or pumped (Jeffery et al., 2015). Flow-through land-based systems are used to rear all sizes of fish and invertebrates in tanks/aquaria, raceways or earth ponds, having the advantage of higher stocking densities due to the greater water exchange compared to recirculating aquaculture systems (RAS) (Jeffery et al., 2015). The water intake to NIMRD's PAS is pumped from the Black Sea and, before entering the aquarium system, it is stored in a covered settlement tank, for sedimentation and reduction of suspended solids. Additional aeration pumps were used to increase the oxygen content of the water.

Salinity and temperature in the PAS were maintained similar to the environment (average salinity 14-15‰). However, when temperature

dropped below 20°C and the blue crab started to become less active, a heater was introduced into the tank in order to keep the temperature at a constant value of 22.5°C.
Live mussels were introduced in the PAS and the *C. sapidus* specimen was immediately observed feeding actively with the mollusks. Additionally, small fish (Black Sea shad, sprat, anchovy and whiting) were offered as food, being rapidly consumed.

RESULTS AND DISCUSSIONS

Overall adaptation

The *C. sapidus* specimen did not display any indication of stress in captivity, showing good perspectives for adaptation.

Feeding behavior

During daily observations, the blue crab individual was noticed feeding regularly with live Black Sea mussels - *Mytilus galloprovincialis* Lamarck, 1819 - in the tank, by detaching them from the substrate, crushing the shell with its pincers and picking the flesh (Figure 6). Among all the fish species offered as food, the blue crab preferred Black Sea shad - *Alosa tanaica* (Grimm, 1901), while the least preferred species was whiting - *Merlangius merlangu*s (Linnaeus, 1758). Anchovy - *Engraulis encrasicolus* (Linnaeus, 1758) - and sprat - *Sprattus sprattus* (Linnaeus, 1758) - were also accepted and consumed (Figure 7).

Figure 6. *C. sapidus* feeding on mussels (Original photo)

Figure 7. *C. sapidus* feeding on fish (Original photo)

Temperature and salinity

Blue crab growth is regulated by water temperature. Growth occurs when water temperatures are above 15°C (Brylawski & Miller, 2006). Water temperature above 33°C is lethal (Bauer & Miller, 2010). As the specimen was collected at the beginning of the cold season, water temperature in the PAS tank started to drop, the crab displaying an inactive behavior below 20°C. In order to stimulate growth and prevent torpor throughout the winter (Rome et al., 2005), the water in the tank was permanently heated at 22.5°C. Under such circumstances, the crab resumed its normal behavior, moving constantly and feeding regularly.
Water salinity is also important, but requirements vary by life stage. Generally, the optimum for adult blue crabs is 3-15 PSU (Rome et al., 2005), in line with Black Sea normal salinity. Regarding the pH, the tolerance range is 6-8, with less than 6 being lethal (Rome et al., 2005). Throughout the entire experimental period, salinity varied between 12-15 PSU, while pH was constant around 7.

Growth and molting

Growth and development of the blue crab, as in other crustaceans, consist of a series of larval, juvenile, and adult stages during which a variety of morphological, behavioral, and physiological changes occur. These changes are most dramatic when the animal molts (sheds its rigid exoskeleton) permitting growth and changes in body shape (Costlow & Bookhout, 1959). Sexual maturity is reached after 18 to 20 postlarval molts, at the age of 1 to 1½ years. Males continue to molt and grow after they reach sexual maturity. It is generally accepted that females cease to molt and grow (terminal molt) when they mature and mate (Mangum, 1992).
Blue crab molting (ecdysis) is a spectacular phenomenon. The molt cycle is divided into four main stages: inter-molt - when the exoskeleton is fully elaborated, pre-molt, ecdysis and post-molt (Roer & Dillaman, 2018). The entire molting process lasts about four to eight weeks; within 3 hours after the molt, the initiation of calcification takes place. Over the next 9 to 12 hours, the shell has a leathery feel. The crab then becomes stiff and

brittle during the next 12 to 24 hours, and the shell becomes hard after 72 hours (Roer & Dillaman, 2018).
The first signs of ecdysis are represented by a faint outline of the second exoskeleton or new skin forming underneath the old as molting approaches ("white sign"). It usually appears about eight weeks prior to molting. Subsequently, the "pink sign" develops - a pink mark that appears on the crab's backfin, which indicates that it will molt in less than a week. This marks the appearance of the new shell underneath its present hard shell. Finally, the "red sign" develops - indicating a hard crab which will molt in less than two days (Shelley & Lovatelli, 2011).
After 6 months of captivity conditions in NIMRD's PAS, the blue crab specimen started to show the first evidence of ecdysis, namely the "white sign", which indicates that molting would occur in a matter of weeks (Figure 8).

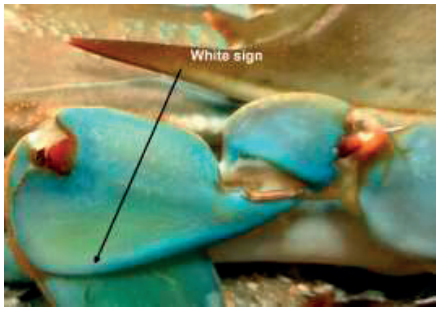

Figure 8. The *C. sapidus* specimen displaying the first mark of ecdysis ("white sign") (Original photo)

Thus, the laboratory conditions under which the blue crab was kept proved to be favorable for a proper development.

Blue crab aquaculture prospects

In recent years, the fishery production of soft-shell crabs has suffered from frequent and significant fluctuations, mainly due to irregular crab fishery landings. About 73.5% of commercial-scale blue crab production originates from wild-caught animals, resulting in large variations in seasonal supply and commercialization of individual specimen size (FAO, 2016). Natural stocks are very vulnerable to climatic and environmental changes and, particularly, to commercial fishing pressure. Regardless of the fishing method, either trawling or trapping, the crab industry dependence of natural stocks is deemed unsustainable in the medium- and long-term (Hungria et al., 2017). Uncontrolled fishing and environmental degradation were reported as the main causes behind the 70% reduction recorded in the *C. sapidus* populations in the Chesapeake Bay (US), once one of the most productive crab fishing areas in North America (Zohar et al., 2008). In 2002, experiments in Chesapeake Bay (USA) were conducted to study the feasibility of blue crab stock enhancement. During 4 years of work, over 290,000 cultured crabs were experimentally released into the bay's nursery habitats, and increased local populations at release sites by 50-250% (Zohar et al., 2008).
The only solution to overcome the dependence on natural stocks for blue crab industry is aquaculture. Commercial crab aquaculture is practiced only in the US and Asia (Hungria et al., 2017). The first experiments to keep crabs in captivity were conducted in America more than 150 years ago, using a system of cages (Perry et al., 1982). In southwestern Asia, the first trials began 50 years later, involving animals in enclosures (Keenan & Blackshaw, 1997). In more recent decades, closed systems using water recirculation (RAS) were developed for crab culture, both in the US and Asia (Hungria et al., 2017). Despite the higher costs associated with the installation and operation of closed systems, they allow greater control over environmental factors, facilitate animal handling and restraint, and minimize mortality losses (Perry et al., 1982).
Large-scale production of larvae and juveniles of *C. sapidus* in captivity is technically possible, although the final survivorship rates and overall results of the process are still unsatisfactory. The main obstacles are the excessive losses due to dietary and nutritional problems, as well as the high rates of cannibalism (Zmora et al., 2005).
At present, the major common points among the main systems currently used for blue crab farming are the confinement of the animals in the pre-molt stage and the requirement that the place used to keep the animals allows an easy monitoring of the ecdysis, as well as fast removal of the recently molted animals. Based on these common features, soft-shell blue crab farming systems can be divided into three groups: *open systems*, carried out in continuous

coastal areas such as bays, coves or lagoons; *semi-closed systems*, undertaken in ponds, similar to those used for fish and shrimp farming; and *closed systems*, carried out in sheltered places and under strict control of environmental conditions (dos Santos Tavares et al., 2017). Open and semi-closed systems represent a more traditional form of cultivation and still widely used to produce *C. sapidus* crabs in the US (Oesterling, 1995). However, in recent years, industrial scale swimming crab production has focused on closed production systems (Gaudé & Anderson, 2011).

Open systems

Enclosure farming represents the most primitive and least technical method to obtain soft-shell swimming crabs, among the systems currently used. Initially, the enclosures used in the production of crabs were circular shaped and constructed with vertically arranged stakes or thin plates of wood and nailed together to prevent the crabs from escaping. A more recent development of this traditional enclosure system has been the installation of individual floating boxes or cages to protect the swimming crabs from cannibalism and predator action (dos Santos Tavares et al., 2017).

Production on an industrial scale requires the installation of thousands of floating cages, which end up occupying a large area (Oesterling, 1955). The difficulty of access seems to be the greatest disadvantage, as the management requires the use of boats, generally involving labor discomfort associated with the handling of the cages (Figure 9) (Gaude & Anderson, 2011).

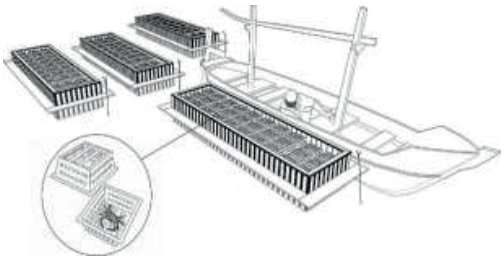

Figure 9. Open system for blue crab farming: routine work of the identification of ecdysis occurrence (after dos Santos Tavares et al., 2017)

Semi-closed systems

Few changes occurred in the production systems until 1950, when a new system was developed. The floating cages were placed inside aquaculture ponds built on land, filled with water pumped from an adjacent brackish or saltwater source and returned to the environment after use (Oesterling, 1995). The ponds currently used are rectangular, with an average area of 100-200 m^2, with the bottom covered with a layer of mud or sand and mud (Figure 10). The animals are kept in small individual cages supported on floating systems, similar to those used in open systems (Oesterling, 1995). The cages are installed in long and narrow floating structures arranged side by side. To ease the management of the cages and the identification of molt, a walkway structure similar to a bridge, usually built of wood, is installed (dos Santos Tavares et al., 2017). Despite advances in water quality control, the system still depends on the existence of salty/brackish water in conditions close to ideal. Moreover, compared with open systems, the ponds involve higher construction and operational activity costs (dos Santos Tavares et al., 2017).

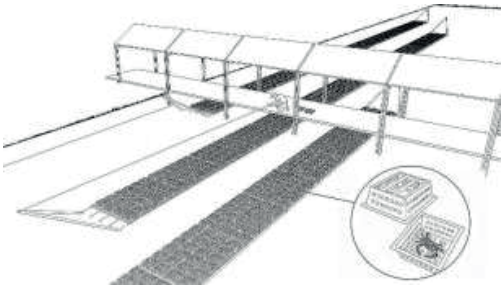

Figure 10. Semi-closed system of the production of soft-shell swimming crabs (after dos Santos Tavares et al., 2017)

Closed systems

The closed system represents the most modern form of blue crab production. The main characteristic is the use of recirculation systems, where water flows through the animal maintenance structures and then through filtration equipment or structures (mechanical, biological and chemical) before returning to the production system (dos Santos Tavares et al., 2017).

The maintenance structures used in closed systems can be communal or individual (cell compartments). Several types of tanks built of wood, concrete, polyethylene or fiberglass can

be used as communal structure (Oesterling, 1995). Cell structures, in turn, involve water circulation through overlapping boxes, cages or drawers (Figure 11) (Shelley & Lovatelli, 2011). This type of production system offers several advantages over the traditional methods above mentioned, such as ensuring a greater control over environmental and operational variables; significantly increasing the availability of locations for the installation of production units; allowing the adoption of high stocking densities; and enabling a better monitoring of the occurrence of ecdysis, aside from allowing several forms of automation (Gaude & Anderson, 2011; Shelley & Lovatelli, 2011).

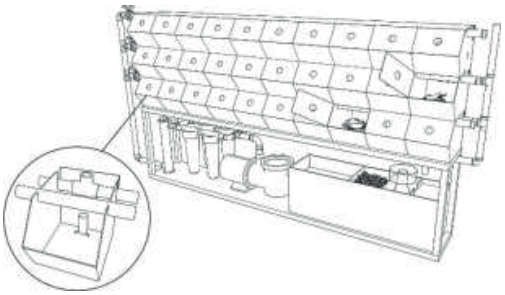

Figure 11. Closed system of cell compartment type for the cultivation of soft-shell swimming crabs (after dos Santos Tavares et al., 2017)

However, closed systems are more complex, requiring more skilled labour and greater investment and production costs (Oesterling, 1995). For instance, there are currently on the market several equipments for recirculating water indoors, including some complete cell systems specific for the production of soft-shell swimming crabs. A system with a capacity for 100 animals can be purchased, directly from specialized online sites, at prices between 10000 and 15000 US dollars (Zhongkehai, 2020).

The animals used in the production of soft-shell swimming crabs are mainly obtained through the capture of individuals in the pre-molt stage in the natural environment and then kept in captivity until molting (dos Santos Tavares et al., 2017). The decrease in the fishing supply of swimming crabs has motivated the research aiming the development of breeding techniques, larviculture and fattening of crabs, under controlled environmental conditions in recent years (Zohar et al., 2008). Large-scale production of larvae and juveniles of *C. sapidus* in captivity is technically possible, although the final survivorship rates and overall results of the process are still to be investigated. The first successful attempt at mass producing juvenile blue crabs was completed in 2005, by researchers at the Center for Biotechnology, University of Maryland Biotechnology Institute (Zmora et al., 2005). Although larvae and juveniles commercial production is still in its incipient stage, blue crab aquaculture is definitely a development to be investigated in the future.

Given the good adaptability to laboratory conditions of the *C. sapidus* specimen, further research of its aquaculture potential at the Romanian Black Sea coast is a promising prospective activity for species diversification.

CONCLUSIONS

The overall behavior of the *C. sapidus* specimen kept in NIMRD's aquaculture laboratory did not indicate signs of stress, showing good perspectives for adaptation. The blue crab individual was observed feeding regularly with live Black Sea mussels. Moreover, small fish (Black Sea shad, anchovy, sprat etc.) were accepted as food. In order to stimulate growth and prevent torpor throughout the winter, the water in the PAS tank was permanently heated at 22.5°C, the crab moving constantly and feeding regularly. As a follow-up, after 6 months under controlled conditions in the PAS tank, the crab started to show the first mark of ecdysis, namely the "white sign", which indicates that molting would occur in a matter of weeks. As such, we can conclude that the laboratory conditions under which the *C. sapidus* specimen was kept proved to be favourable for a proper development. The blue crab individual shall be further monitored, in order to accurately document the molting process.

However, given that soft-shell crab aquaculture is a costly (high prices for equipment, manpower, utilities etc.) and technologically demanding endeavour (temperature control, appropriate feed provision, skilled staff for ecdysis monitoring etc.), a socio-economic and feasibility study should be performed, in order

to assess its applicability to the Romanian Black Sea area. This study must take into account that the revenues generated by selling wholesale soft-shell crabs can reach more than 20 US dollars/kg. Moreover, with a proper marketing of the blue crab as a luxury dish in restaurants, it can be advertised as a niche product, which would certainly be accepted and generate income.

Additionally, whereas our research has highlighted the most critical aspects for *C. sapidus* rearing in captivity (temperature, salinity, feeding preferences etc.), the blue crab can be an excellent candidate for the aquarium business (either public, for educational purposes, or for marine aquaria hobbyists). Records indicate that adult caught crabs survived in captivity for more than 1 year, thus, under proper culture conditions, they can be an attractive species.

In the future we intend to create a pilot system to raise blue crabs.

ACKNOWLEDGEMENTS

This study has been carried out with financial support from the NUCLEU INTELMAR Programme, funded by the Romanian Ministry of Education and Research, project no. PN 19260301, and the GFCM, through the Shellfish Aquaculture Demonstrative Center (S-ADC).

The authors would also like to thank Mr. Aurel Amzaru for kindly providing the blue crab specimen.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

EFFECT OF DIETARY VITAMIN C ON THE HAEMATOLOGICAL PROFILE OF JUVENILE EUROPEAN CATFISH (*SILURUS GLANIS*) REARED INTO RECIRCULATING SYSTEM

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Abstract

A feeding trial was conducted to evaluate the effects of dietary vitamin C (L-ascorbic acid, AA) levels on haematological profile and some biochemical indices of European catfish, reared in a recirculating aquaculture system. A basal commercial diet (40% crude protein and 11.5% lipids) was used as a control (D_0), and three other diets were prepared by supplementing the feed with 50 mg AA kg^{-1} diet (D_1), 100 mg AA kg^{-1} diet (D_2) and 150 (D_3) mg AA kg^{-1} diet. At the end of the feeding trial, blood samples were taken in order to analyse the haematological profile and serum biochemical parameters. Significant differences ($p<0.05$) were recorded among experimental diets in the numbers of red blood cells (RBC), haematocrit (Hct), the mean corpuscular volume (MCV), and the mean corpuscular haemoglobin concentration (MCHC). Regarding the serum glucose, serum total protein, and the concentration of immunoglobulin (IgM) it was observed an insignificant increase ($p>0.05$) with the increasing of the level of vitamin C. In conclusion, supplementation of vitamin C in the diet of Silurus glanis led to good results on fish welfare.

Key words*: Silurus glanis, Vitamin C, hematological profile, serum parameters.*

INTRODUCTION

According to Food and Agriculture Organization (2018), aquaculture continues to be the fastest-growing food sector worldwide. However, aquaculture continues to facing some issues such as diseases (Rahman et al., 2019), feed contamination, and environmental impacts (Crețu et al., 2016).

From all the factors which influence fish growth performance and welfare, in a recirculating aquaculture system, the quality of feed plays a very important role in fish metabolism and welfare (Martinez-Porchas & Martinez-Cordova, 2012). So, for fish, essential nutrients such as proteins, essential fatty acids, vitamins C and E, polysaccharides, and some minerals have pivotal importance to response on the growth, haematological and immunological parameters (Barrows et al, 2008).

In the literature, many research indicates that vitamin C is an important micronutrient that plays a significant role in fish growth by enhancing feed conversion efficiency, protein efficiency ratio (Ai et al., 2006; Eo and Lee, 2008; Alam et al., 2009; Cocan et al., 2018; Dicu et al., 2013), physiological stress (Farahi, 2012), improvement of some haematological parameters like plasma proteins, red blood cell (RBC) count, haematocrit (Hct) value and white blood cell (WBCs) count (Wang et al., 2003; Zhou et al., 2003; Affonso et al., 2007; Nsonga et al., 2009; Pimpimol et al., 2012).

Vitamin C must be supplied in the fish diet, because most of the fish species are unable to synthesize vitamin C since they do not have the enzyme L-gluconolactone oxidase which is responsible for synthesis from glucose (Oprea & Georgescu, 2000; Dabrowski, 2000; Trichet, 2015). Also, addition of vitamin C in fish diet proves to reduce the toxic effects of environmental pollutants on fish organism. Therefore, increasing the bioavailability of vitamin C may reduce the effects of environmental toxins on fish (Mehrpak et al., 2015).

The quantity of vitamin C which has to be added to the fish diet varies according to fish species, size, feeding rates, environmental

factors, nutrient interrelationships, health condition, water quality, feed formulation technique (Gouillou-Coustans and Kaushlic, 2000), culture conditions (NRC, 2011) respectively form of the vitamin C that is supplied (National Research Council, 2011). The addition of some concentrations of AA in the diet enhances the immune status and disease resistance in channel catfish (Li Y. et al., 1985), Indian major carp (Sahoo et al., 2003), rainbow trout (Fazaei et al., 2015), Japanese sea bass (Ai et al., 2004), Nile Tilapia (Mirea et al., 2013), Stellate sturgeon (Dicu et al., 2013), goldfish (Nica et al., 2016).
Therefore, the objective of this study was to evaluate the effects of dietary vitamin C levels on the haematological profile and some biochemical indices of European catfish reared in a recirculating aquaculture system.

MATERIALS AND METHODS

Experimental trial was conducted at the "Dunărea de Jos", University of Galați, in a recirculating aquaculture system (RAS). The RAS system was previously described in our earlier studies (Enache et al., 2011).
The experiment was conducted during five weeks period. 123 fish juveniles with an average weight of 118.96±0.43 g were randomly distributed to the rearing units of the RAS. The food used for the biomass culture was extruded pellets with a diameter of 4.3 mm an adequate content for the age of the fish (Table 1).

Table1. Chemical composition of feed

Parameter		Quantity
Crude protein		40%
Crude fat		11.5%
Crude ash		7.5%
Crude cellulose (fiber)		4%
Phosphorus		0.7%
Vitamin A		3000 (UI/kg)
Vitamin D		600 (UI/kg)

The ratio used during this experiment was 2% of the fish's body weight. The daily ratio was divided into two equal parts and was fed in the morning and in the evening
Four experimental groups were carried out: D_0-commercial feed, without vitamin C; D_1-commercial feed supplemented with 50 mg vitamin C kg^{-1}; D_2-commercial feed supplemented with 100 mg vitamin C kg^{-1} and D_3-commercial feed supplemented with 150 mg vitamin C kg^{-1}. Ascorbic acid (AA) used was provided from Janssen Chimica Company (Geel, Belgium) and had 99.9% purity. The incorporation technique of AA in commercial feed was described by Plăcintă et al., 2012.

Water quality. The water quality was monitored during the experiment. Dissolved oxygen and temperature were measured daily with Hanah HI 9147-04, the pH was measured with pH meter WTW model 340. One time per week N-NH_4^+, N-NO_2^-, N-NO_3^- concentrations were determined with Spectroquant Nova 400 photometer compatible with Merk kits.

Blood sampling and analysis. Fishes were anesthetized using 2-phenoxyethanol bath (8 ml 40 L^{-1} of water for 5 minutes) before taking blood from the fish. For blood collection, samples were taken at ten fish from each experimental variant from vena caudalis using syringes that previously were rinsed with lithium heparin. For serum collection, blood samples were taken without using anticoagulant and then transferred immediately to Eppendorf tubes. Blood samples taken were centrifuged at 3,500 rpm for 10 min to obtain serum.
The red blood cell counts (RBC × 10^6 μL^{-1}) were determined with Neubauer hemocytometer using a Potain pipette and Vulpian diluting solution.
For the haematocrit (Ht, %) determinations, blood samples (30 µL) were put in micro-haematocrit capillary tubes and then centrifugated for 5 min at 10,500 rpm. Measurements were made by microhematocrit reader and expressed as a percentage.
Haemoglobin (Hb, g dL^{-1}) concentration was determined by the cyanmethaemoglobin method by adding 20 µl of whole blood to 5 ml of Drabkins solution. The absorbance was measured using a spectrophotometer (Specord 210-Analytic Jena), at a wavelength of 540 nm.
The mean corpuscular volume (MCV, μm^3), the mean corpuscular haemoglobin (MCH, pg), and the mean corpuscular haemoglobin concentration (MCHC, g dL^{-1}) were calculated from the values of Ht, Hb, and RBC, according to Ghergariu et al. (1985).

Plasma glucose levels (GLU, mg dL^{-1}) was determined by the glucose oxidase method using a UV-Vis spectrophotometer at a wavelength of 635 nm.
Total protein (TP, g dL^{-1}) was determined according to the Biuret method. The absorbance was read at 546 nm wavelength. The immunoglobulin (IgM, mg dL^{-1}) levels were determinate by the method of Bakopoulos (1997).
Lipid peroxidation or malondialdehyde-MDA, (nmol mL^{-1}) level was determined from tissue, liver, kidney, intestine, and blood plasma, according to Draper & Hadley (1990) method, at an optical density of 532 nm.

Statistical analysis. All data are presented as mean ± standard deviation. Data were subjected to ANOVA test using SPSS 21 version. Before ANOVA, the normality of the data was verified by Kolmogorov-Smirnov test. When the ANOVA reveals a significant difference ($p<0.05$), Tukey's test was used for post-hoc multiple comparisons.

RESULTS AND DISCUSSIONS

The results obtained in our experiment for the haematological profile were corroborated with those of fish growth performance (Plăcintă et al., 2012). So, from the technological data at the end of the experiment, it was concluded that fish from the V_1 variant registered a better growth performance. The final weight of fish from the D_1 variant (172.52 g) was significantly higher ($p<0.05$) than those from the control variant (164.22 g), or from V_2 (162.86 g) or V_3 (164.67 g).
Also, in a RAS system water quality have a crucial role and any problems associated with it will result in deterioration of fish health. During the experiment, the water parameters remained in the normal ranges for the species (Bhatnagar et al., 2013) and showed no significant differences (ANOVA, $p>0.05$) across treatment diets. The means values of dissolved oxygen, temperature, and pH were 5.24±0.44 mg L^{-1}, 20.29±1.13ºC, respectively 7.77±0.14 pH units. Regarding the nitrate, nitrite, and ammonium concentrations the means values were 76.24±5.22 mg L^{-1}, 0.56±0.09 mg L^{-1}, respectively 0.77±0.12 mg L^{-1}.

In figures, 1-6 are presented the obtained values of the haematological parameters at the end of the experiment. The results obtained showed significant differences ($p<0.05$) in terms of the number of erythrocytes, haematocrit percentage, mean corpuscular volume, and the mean corpuscular haemoglobin concentration.
The *red blood cell count (RBC)* showed significant differences between the experimental groups (ANOVA, $p<0.05$).
Thus, the post hoc analysis of Tukey's showed that the values of erythrocytes from the control group (1.58±0.06 × 10^6 cells μL^{-1}) were not statistically different from those obtained in D_1 (1.52±0.12 × 10^6 cells μL^{-1}). Significantly higher values of RBC were registered in the D_2 (1.74±0.09 × 10^6 cells μL^{-1}) and at the D_3 variant (1.67±0.08 × 10^6 cells μL^{-1}) (Figure 1).

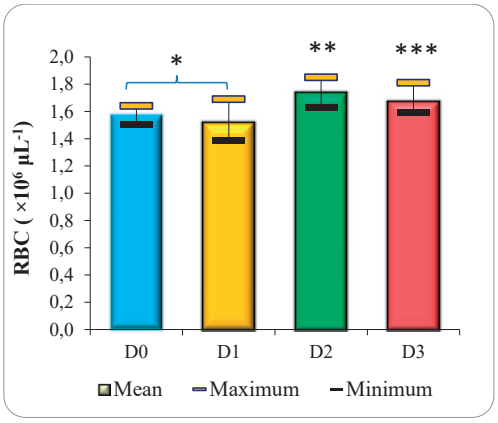

Figure 1. Mean, minimum and maximum values of RBC erythrocytes of *S. glanis*

Haematocrit percentage (Ht) showed significant differences ($p<0.05$) between the experimental variants. The haematocrit percentage from the variant D_3 (27.86±0.69%) was significantly higher than D_2 (26.20±0.78%), while no differences were recorded between the control group (22.02±0.6%) and D_1 (21.66±0.58%) (Figure 2).
The *haemoglobin concentration (Hb)* showed no statistical differences between the experimental variants. However, a slight increase in haemoglobin concentration was registered in the D_3 variant (9.10±0.27 g dL^{-1}), while in the D_0, D_1, and D_2 the mean value of the haemoglobin concentration was 8.64±0.39

g dL^{-1}, 8.42±0.6 g dL^{-1}, 8.52±0.46 g dL^{-1} (Figure 3). Analysing the results of the erythrocyte constants, it can be observed that the administration of vitamin C in feed has induced significant differences ($p<0.05$) in MCV and MCHC.

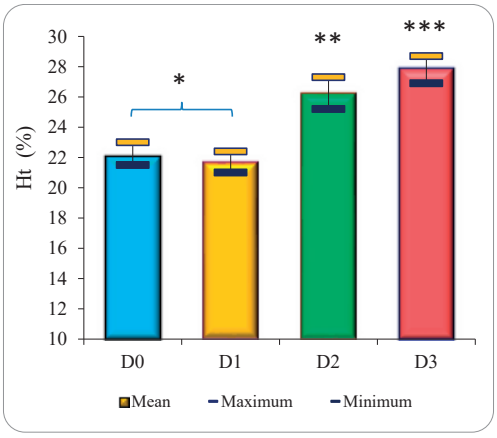

Figure 2. Mean, minimum and maximum values of the hematocrit of *S. glanis*

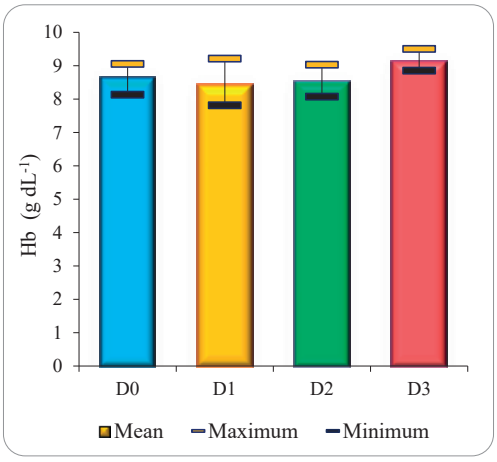

Figure 3. Mean, minimum and maximum values of haemoglobin of *S. glanis*

Regarding *the mean corpuscular volume (MCV)* values the post hoc analysis of Tukey's test showed that the MCV value from D_3 (167.21±1.02 μm^3) was significantly higher in comparison with those from the D_0 (139.63±8.91 μm^3), D_1 (143.56±11.31 μm^3), and D_2 (154.94±5.43 μm^3) (Figure 4).

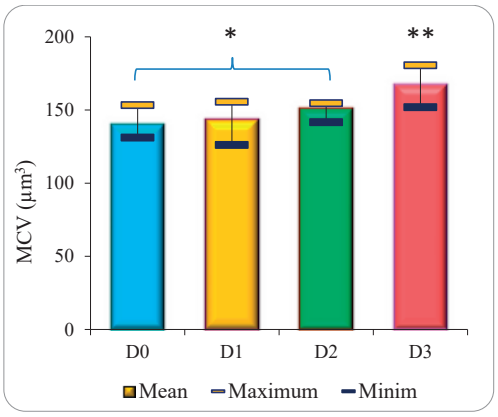

Figure 4. Mean, minimum and maximum values of MCV of *S. glanis*

The *mean corpuscular haemoglobin (MCH)* indicates the erythrocyte loading with haemoglobin. The MCH values were 54.67±0.49 pg in D_0, 55.95±7.04 pg in D_1, 49.20±4.92 pg in D_2, and 54.60±3.16 pg in D_3, without significant differences (ANOVA, $p>0.05$) between the four experimental variants (Figure 5).

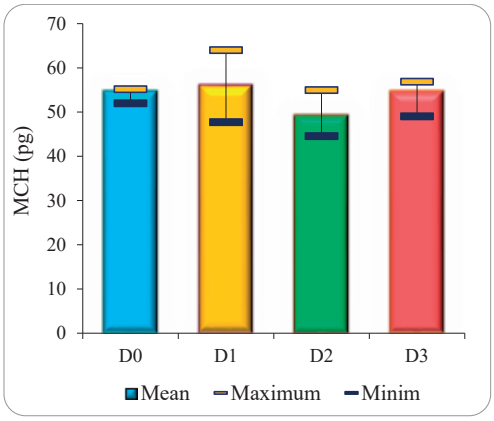

Figure 5. Mean, minimum and maximum values of MCH of *S. glanis*

The mean corpuscular haemoglobin concentration (MCHC) was significantly (ANOVA, $p<0.05$) influenced by the administrated concentration of vitamin C. The post hoc analysis showed that the values of MCHC from the D_2 (32.57±2.66 mg dL^{-1}) and D_3 (32.68±1,17 mg dL^{-1}) were significantly lower than those from D_0 (39.29±2.72 mg dL^{-1}) and D_1 (38.95±3.50 mg dL^{-1}) (Figure 6).

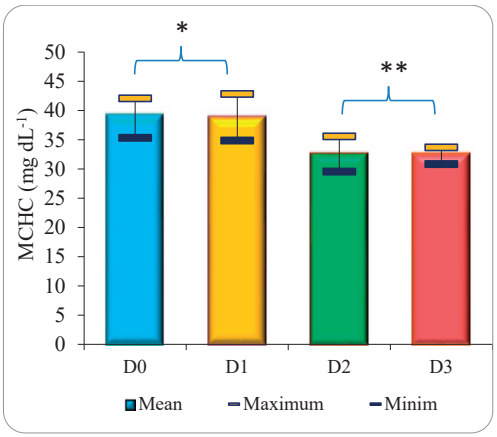

Figure 6. Mean, minimum and maximum values of MCHC of *S. glanis*

Haematological analyses may provide a reflection of the health status of fish (Docan et al., 2011). In our experiment the haematological results obtained for *Silurus glanis* are in the line with those presented in the literature (Table 2).

Table 2. Normal values of haematological profile of *Silurus glanis*

Haematological parameters	Docan et al., 2010	Docan et al., 2011
RBC (10^6 µL^{-1})	1.36 ± 0.17	1.41±1.77
Ht (%)	22.30 ± 2.7	27.1±2.66
Hb (g dL^{-1})	7.33 ± 0.88	6.82±0.73
MCV (µm^3)	165.66± 5.06	194.28±24.4
MCH (pg)	54.43 ± 8.05	48.67±4.66
MCHC (mg dL^{-1})	32.97 ± 2.38	25.25±2.5

In our study, the administration of vitamin C in the *Silurs glanis* food led to the improvement of the haematological profile. Similar results were obtained by Falatkar (2005) in the case of *Huso huso.* Also, Pimpimol et al. (2012), reported in the case of Mekong giant catfish (*Pangasianodon gigas*) an improvement of haematological profile when the fish feed was supplemented with 500 and 750 mg kg^{-1} vitamin C of feed. According to Sahoo and Mukherjee, (2003) increasing RBC values is due to the powerful antioxidant action of vitamin C that protects various tissues of fish, including RBC, against oxidative damage. Since RBC are involved in the control of immune functions (Madhusudan et al., 2015) and are the main production sites of free radicals, the increase of the number of erythrocytes can be attributed to the administration of vitamin C, respectively to the improvement of the oxidative state of fish. Also, according to Dinning (1962), the addition of vitamin C in the fish diet led to the increase of erythropoiesis.

In Table 3 are presented the mean values±SD for the blood serum at the end of the experimental period.

Table 3. The values of glucose, total proteins, and immunoglobulins at the end of the experimental period

Exp. var.	GLU (mg dL^{-1})	TP (g dL^{-1})	IgM (mg dL^{-1})
D_0	69.80±6.18	3.60±0.20	13.55±0.54
D_1	77.40±3.78	3.46±0.28	13.46±0.72
D_2	74.40±3.97	3.56±0.30	14.03±0.52
D_3	73.40±6.66	3.73±0.22	14.03±0.35
ANOVA	0.26	0.52	0.47

In the present study, blood glucose, total proteins, and immunoglobulins levels showed no significant changes ($p<0.05$) between the experimental variants. However, it was observed a slightly decreasing trend of serum glucose with the increasing concentration of vitamin C in the fish feed, while the TP and IgM, showed a slight decrease.

According to some authors addition of high quantities of vitamin C in fish feed can enhance protein synthesis (Andrade et al., 2007). Pimpimol et al., 2012 reported also a slightly increased after 8 weeks of experiment of total serum protein in Mekong giant catfish fed high vitamin C concentrations (500 and 750 mg vitamin C kg^{-1} feed).

According to Jiang et al. (2013), MDA is widely used indicator of oxidative damage to lipids of cell membrane by lipid peroxidation, which is accompanied by the reduction in antioxidant capacity. Lipid peroxidation evaluations in the present research were determined from muscular tissue, kidney, liver, intestine, and blood serum (Figure 7). Significant differences (ANOVA, $p<0.05$) were recorded between the obtained values in all the experimental variants. The post hoc tests showed that MDA values from kidney and liver from D_1 and D_2 variants were significantly ($p<0.05$) lower than those from D_0 and D_3.

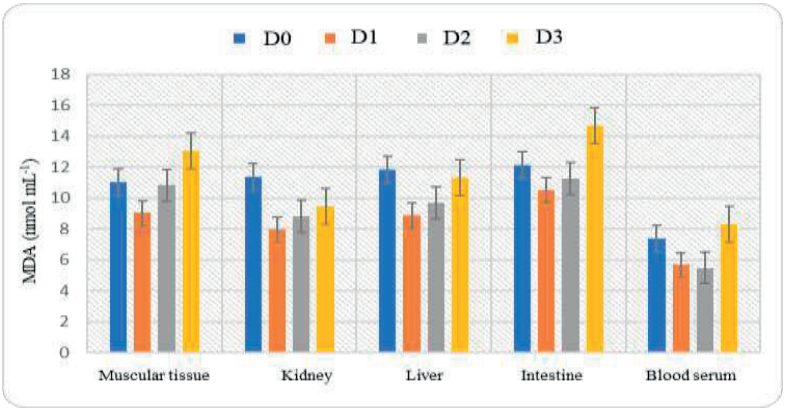

Figure 7. The mean values ± SD of MDA for muscular tissue, kidney, liver, intestine, and blood serum at the end of the experiment

In the case of MDA from the muscular tissue, intestine and blood serum it was observed an intensification of oxidative stress in the case of the highest dose of vitamin C (D3). Malondialdehyde is widely used as an indicator of lipid peroxidation (Esterbauer et al., 1991) and increased levels of MDA are associated with a variety of chronic diseases (Allen-Gil and Martynov, 1995). The improvement of oxidative stress was obtained at lower concentrations of vitamin C (50, 100 mg kg^{-1}), observing an intensification of MDA at a concentration of 150 mg kg^{-1}. However, the values of lipid peroxidation were within the same range of other fresh water fishes (Antache et al., 2013).

Generally, the use of vitamins as feed additives is recommended in the diets of farmed fish. Several studies reported that the addition of vitamin C on fish feed had a positive effect on the haematological parameters by increasing RBC, Ht, or Hb concentration (Zafar and Khan, 2020).

In our study, a significant increase of RBC and Ht was found with increasing of vitamin C concentration (100 mg AA kg^{-1} diet and 150 mg AA kg^{-1} diet), indicating that vitamin C protected the RBC membranes from oxidation and improved the ability of oxygen transport. Also, a positive effect on oxidative stress was observed in the variants where vitamin C was supplemented in fish feed at concentration of 50 and 100 mg kg^{-1} feed.

CONCLUSIONS

The results of the present research suggested that the dietary incorporation of vitamin C exerts a positive effect on the haematological profile and oxidative capacity at a dose between 50-100 mg kg^{-1}. However, more research is needed in order to determine the optimum dietary requirement of vitamin C for *Silurus glanis* according to the age and size of the fish, or the breeding system.

ACKNOWLEDGEMENTS

The authors are grateful for the technical support offered by MoRAS through the Grant POSCCE ID 1815, cod SMIS 4874 (www.moras.ugal.ro).

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

INVESTIGATION ON PARASITOFAUNA OF SOME FRESHWATER FISH FROM SUPERIOR AND MIDLE AREA OF ROMANIAN DANUBE RIVER SECTOR

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Abstract

During the years 2019-2020, research was carried out on the assessment of the health status of fish in the upper Danube sector of Romania. Fish species belonging to the families Ciprinidae, Siluridae, Esocidae, Percidae, Clupeidae and Acipenseridae were analyzed. Fish were sampled from two stations: Station 1- km 1048, near Moldova Nouă and Station 2- km 493, near Giurgiu. The parasitological analyzes were performed in the laboratory of the Institute of Research and Development for Aquatic Ecology, Fishing, and Aquaculture (ICDEAPA), Galati, Romania. Analyzes were performed on fresh fish using well-known methods. In station 1, 14 species of parasites belonging to eight systematic groups were identified: Nematode, Monogenea, Trematode, Ciliata, Acanthocephala, Protozoa, Cestoda, Crustacea, while in station 2, 11 species of parasites belonging to six systematic groups were identified: Monogenea, Ciliata, Trematoda, Nematoda, Acanthocephala, Cestoda. The species Sander lucioperca and Abramis brama presented most often polyparasitoses but with a low degree of infestation. The most present group of parasites in station 1 was represented by nematodes and in station 2 by monogenic worms.

Key words*: Danube river, fish parasites, parasitological analysis, wild fish.*

INTRODUCTION

In recent decades, aquatic ecosystems have been subjected to increasing anthropogenic pressure. Aquatic organisms are frequently exposed to several stressors, natural and artificial, such as physical variations and chemical parameters of the environment (rainfall, temperature, and salinity), changes in food and habitat availability, and increased exposure to contaminants. in nutrient intake (eutrophication) (Adams & Greeley, 2000).

Therefore, the methodologies that increase our understanding of these phenomena of pressure on aquatic organisms have great importance.

To assess and quantify the effect of environmental stressors on the health of aquatic systems, researchers used bioindicators, defined as organisms or communities whose vital functions are so closely correlated with certain environmental factors that they can be used as indicators in the assessment of a particular area (Markert et al., 2003).

Field approaches are vital for an integrated assessment of these ecosystems, allowing the detection of the cumulative and/or synergistic effects of the impact on the environment and the community of organisms (Adams et al., 1999).

The parasite fauna of aquatic organisms is it is pervasive and is a hidden component of ecological communities, which are closely linked to several characteristics of the biotic and the abiotic environment in which they live.

Thus, fish parasites have attracted increasing interest from researchers as potential indicators of environmental quality, due to the variety of forms that respond to anthropogenic pollution, such as eutrophication, oil spills, heavy metals, acid rain, sewage leaks, agricultural and industrial pollution (Landsberg et al., 1998; Sures, 2004).

The effects of stressors on parasite communities are varied and can be positive or negative: pollution can increase parasitism and can be fatal for certain species, leading to a decrease in the number of parasites. Abiotic

factors such as temperature, dissolved oxygen, salinity, and pH can influence the appearance of parasites temporally and spatially, especially helminth parasites of fish (Chubb, 1979)

Stressors can promote parasitism, for example, if the host's defense mechanisms are adversely affected, thereby increasing the host's susceptibility, or simply by increasing the density on the final and intermediate host, such as eutrophication, usually favors invertebrates as intermediate hosts in the life cycle of digenic helminths (Sures, 2004). For example, eutrophication can increase parasitism, while heavy metals can reduce it. Ciliates and nematodes are sensitive indicators of eutrophication and thermal effluents, while digens worm and acanthocephalus are good indicators of heavy metals (Lafferty, 1997). Poulin (1992) showed that parasite fauna is indirectly influenced by pollutants that are toxic to fish and intermediate hosts and directly by environmental factors that are toxic to parasites and their free life forms.

The present paper has the role of highlighting an image of the fish parasite fauna from the upper and middle sectors of the Danube river km 1048 - Moldova Nouă area and km 493 - Giurgiu.

MATERIALS AND METHODS

Over the years 2019-2020, we sampled the fish community from the upper and middle Danube River sector (Moldova Nouă area km 1048 and Giurgiu area km 493) belonging to the following species: *Carassius auratus gibelio* (Bloch, 1782), *Hypophthalmichthys molitrix* (Valenciennes, 1844), *Cyprinus carpio* (Linnaeus, 1758), *Abramis brama danubii* (Linnaeus, 1758), *Rutilus rutilus carpatorossicus* (Linnaeus, 1758), *Scardinius erythrophtamus* (Linnaeus, 1758), *Leuciscus idus* (Linnaeus, 1758), *Ballenus sapa* (Pallas, 1814), *Alburnus alburnus* (Linnaeus, 1758), *Aspius aspius* (Linnaeus, 1758), *Perca fluviatilis* (Linnaeus, 1758), *Silurus glanis* (Linnaeus,1758), *Esox lucius* (Linnaeus,1758), *Sander lucioperca* (Linnaeus, 1758), *Acipenser ruthenus* (Linnaeus, 1758), *Alosa immaculata* (Bennett,1835).

The fishing area was represented by a sector of the Danube River with a surface of 16.15 km^2 (L = 9.5 km, l = 1.7 km). The scientific fishing activity was carried out over a length of 2-3 km, with the fishing net wall. The fish were weighed (g) and their total length was measured (cm).

Fish were transported to the Institute of Research and Development for Aquatic Ecology, Fishing and Aquaculture Galați laboratory where parasitological analyses were carried out.

The sampled fish were examined for both ectoparasites and endoparasites using standard parasitological procedures. The taxonomic classification and identification of the observed parasites were done based on Munteanu & Bogatu (2003), Bauer (1984), Bauer (1985), Bauer (1987). The external surface of the fish was examined thoroughly using a hand lens. Areas around the fins, nostrils, operculum, and the buccal cavity were examined for external parasites (monogeneans and crustaceans). Each fish was opened dorso-ventrally and its internal organs were examined for parasites. The entire digestive system was removed and placed in a Petri dish with physiological saline, and the gut was divided into sections. For isolation, selection, and identification of the parasite fauna of wild fish from the Danube river, we used a Zeiss microscope. We also analyzed the extensity and intensity of parasitic infestation of the fish specimens according to Bush (1997).

RESULTS AND DISCUSSIONS

In Station 1, area Moldova Nouă km 1048, 15 species grouped in 5 families were captured: Cyprinidae, Esocidae, Percidae, Siluridae, Acipenseridae. From the 15 species captured, lots were set up for ichthyo-pathological research (3-5 fish) of the following fish species: *Carassius auratus gibelio* (gibel carp), *Hypophthalmichthys molitrix* (silver carp), *Cyprinus carpio* (carp), *Abramis brama danubii* (carp bream), *Rutilus rutilus carpatorossicus* (roach), *Scardinius erythrophtamus* (common rudd), *Leuciscus idus* (ide), *Ballenus sapa* (White-eye bream), *Alburnus alburnus* (bleak), *Aspius aspius* (asp), *Acipenser ruthenus* (sterlet), *Perca fluviatilis* (European perch), *Silurus glanis* (catfish), *Esox lucius* (pikepeach), *Stizosteidon lucioperca* (zander).

Table 1. Species of parasites identified in the upper Danube River sector km 1048 (Moldova Nouă area)

No. crt.	Identified parasitic species	Parasitic species	Affected organ	Degree of infestation
1.	*Achtheres percarum*	*Sander lucioperca*	G	weak
2.	*Ichthyocotylurus pileatus*	*Sander lucioperca*	I	weak
3.	*Apophallus donicum*	*Sander lucioperca*	T	weak
		Perca fluviatilis	G	weak
4.	*Bunodera luciopercae*	*Perca fluviatilis*	I	weak
5.	*Trichodina domerguei*	*Hypophthalmychtis molitrix*	G	weak
		Cyprinus carpio,	G	weak
		Leuciscus idus	G	weak
6.	*Trichodinella epizootica*	*Sander lucioperca*	G	weak
7.	*Myxobolus macrocapsularis*	*Rutilus rutilus*	G	weak
8.	*Myxobolus obesus*	*Alburnus alburnus*	G	weak
9.	*Dactylogirus vastator*	*Carasus auratus gibelio*	G	weak
		Cyprinus carpio	G	weak
		Abramis brama danubii	G	weak
		Ballerus sapa	G	weak
10.	*Diplozoon paradoxus*	*Abramis brama danubii*	G	weak
		Rutilus rutilus carpathorossicus	G	weak
		Scardinus erythrophthamus	G	weak
11.	*Triaenophorus nodulosus (lucii),*	*Esox lucius*	I	medium
12.	*Eustrongylides excisus*	*Perca fluviatilis*	M	weak
		Sander lucioperca	M	weak
		Silurus glanis	M	weak
		Esox lucius	M	weak
		Aspius aspius	M	weak
13.	*Pomphorhynchus leavis*	*Silurus glanis*	I	weak
14.	*Acanthocephalus anguillae*	*Acipenser ruthenus*	I	weak

Note: T-tegument;G-gills; I-intestine; L-liver; E-eye; M-muscles.

Achtheres percarum, crustacean copepod that was found parasitizing gills on the *Stizostedion lucioperca*. Hypertrophies and agglutination of the gill lamellae were observed, which became fusiform and whitish. The parasite was observed fixed on the pharyngeal teeth.

Pomphorhynchus leavis, is an acanthocephalus that has 18-20 rows of 12 hooks each. It was found in the intestine at catfish (*Silurus glanis),* and barbell (*Barbus barbus*). No intestinal lesions were observed. This parasite is a common acanthocephalus of fish in the Danube Delta, being a typical southern euryhaline form (Docan et al., 2019).

Eustrongylides excisus - nematode with red larvae and clockwise shape, were frequently found in the abdominal cavity and the muscles at *Perca fluviatilis, Stizostedion lucioperca, Silurus glanis*, *Esox lucius,* and *Aspius aspius*. The presence of this parasite is was reported in similar hosts by Cojocaru (2003).

Triaenophorus nodulosus (lucii) cestode, recognized by the pair of three-forked hooks at the level of the scolex it was found at pikeperch (*Esox lucius*). Only at one pikepech fish it was found in autumn ten specimens of parasite without any changes in the general condition of the fish. But the intestinal mucosa has ulcerations and nodules.

Bunodera luciopercae (2-3 mm long trematode with a well-developed anterior triangular suction cup) were found in the gut of perch (*Perca fluviatilis*). The parasites were found in autumn (September-October).

Ichthyocotylurus pileatus - trematode that produces whitish spherical cysts and disseminated on serous (especially on the pericardium, but also on the esophageal wall) to the common shawl (*Stizostedion lucioperca*). The cysts contain a metacercaria which, to be highlighted, must be washed several times after extraction from the cyst.

Apophallus donicum, trematode produces black cysts the size of needle dung scattered on the swimmer, but also the epidermis. They identified themselves at the shawl *(Stizostedion lucioperca)* and perch *(Perca fluviatilis)* fish from the Danube. It is one of the most widespread parasitic diseases, both in freshwater fish and saltwater, and is caused by *Trichodina* species (Totoiu, 2018)

Trichodinella epizootica - ciliated - identified in the gill scrapes performed at the shawl *(Stizostedion lucioperca).*

Trichodina domerguei - ciliated - identified in the gill scrapes of the species: *Hypophthalmychtis molitrix, Cyprinus carpio* and *Leuciscus idus* from the study area.

Myxobolus. macrocapsularis and *Myxobolus obesus* are protozoa that produce whitish cysts, elongated by 1-2 mm disseminated on the gill lamellae in the roach (*Rutilus rutilus*) and bleak (*Alburnus alburnus*), respectively.

Of the total specimens that constituted the lots for the parasitological examination, 79% have weak poly-parasitosis, of which: 25% produced by nematodes (*Eustrongylides excisus*), 24% by monogenic worms (*Dactylogirus vastator* and *Diplozoon paradoxus*), 14% by trematods (*Bunodera luciopercae, Ichthyocotylurus pileatus, Apophallus donicum*), 16% by ciliated (*Trichodina domerguei* and *Trichodinella epizootica*), 11% by acanthocephalus (*Pomphorhynchus leavis and Acanthocephalus anguillae*), 5% by protozoa (*Myxobolus. macrocapsularis and Myxobolus obesus*), 2.5% by cestode (*Triaenophorus nodulosus* (lucii), 2.5% by copepods (*Achtheres percarum*).

Table 2. Species of parasites identified in the middle Danube River sector km 493 (Giurgiu area)

No. crt.	Identified parasitic species	Parasitic species	Affected organ	Degree of infestation
1.	*Trichodina domerguei*	*Carasus auratus gibelio*	G	weak
		Sander lucioperca	G	weak
		Cyprinus carpio	G	weak
2.	*Trichodinella epizootica*	*Abramis brama*	G	weak
		Sander lucioperca	G	weak
3.	*Dactylogirus vastator*	*Carasus auratus gibelio*	G	weak
		Cyprinus carpio	G	weak
		Abramis brama	G	weak
4.	*Diplozoon paradoxus*	*Abramis brama*	G	weak
		Rutilus rutilus	G	weak
		Scardinus erythrophthamus	G	weak
5.	*Diplostomum spathaceum*	*Rutilus rutilus*	E	weak
		Hypophthalmychtis molitrix	E	weak
6.	*Ligula intestinalis*	*Cyprinus carpio*	I	weak
		Rutilus rutilus	I	weak
7.	*Myxobolus carassi*	*Carasus auratus gibelio*	G	weak
8.	*Allocreadium isoporum*	*Scardinius erythrophtalmus*	I	weak
		Abramis brama danubii	I	weak
9.	*Contracecum aduncum*	*Alosa immaculata*	I	medium
10.	*Mazocraes alosae*	*Alosa innaculata*	G	weak
11.	*Pomphorhynchus leavis*	*Silurus glanis*	I	weak
		Barbus barbus	I	medium

Note: T-tegument; G-gills; I-intestine; L-liver; E-eye; M-muscles.

At the Station 2 of the Danube River km 493 - Giurgiu area - 10 species of fish grouped in 4 families were caught: Cyprinidae, Percidae, Siluridae, Clupeidae. Drom the ten species caught, lots were set up for ichthyo-pathological research (3-5 specimens) of the following fish species: C*arassius auratus gibelio* (gibel carp)*, Hypophthalmichthys molitrix* (silver carp)*, Cyprinus carpio* (carp), *Abramis brama danubii* (carp bream), *Rutilus rutilus carpatorossicus* (roach), *Scardinius erythrophthalmus* (common rudd)*, Barbus barbus* (common barbel), *Silurus glanis* (catfish) and *Alosa immaculata* (Pontic shad).

Allocreadium isoporum it was found in intestine at common rudd (*Scardinius erythrophtalmus*) at the begging of the summer. According to Cojocaru (2003) the adults probably die after laying eggs, and larval development does not end until the following spring. They are small trematodes (0.7 mm long).

Mazocraes alosae belongs to the class of monogenic flatworms and was found weakly parasitizing (5-10 specimens/fish) in the gills of Pontic shad.

Contracecum aduncum belongs to the class of nematods and was found in the intestine of Danube ash trees in the number of 30-50 specimens/intestine.

Lingula intestinalis, belongs to the class of cestoses and were found in spring and summer, weakly parasitizing the intestine of cyprinids.

Of the total specimens that constituted the lots for the parasitological examination, 87% have weak and medium polyparasitosis, from which: 35% by monogenic worms (*Dactylogirus vastator, Mazocraes alosae* and *Diplozoon paradoxus*), 30% by cilliated (*Trichodina domerguei* and *Trichodinella epizootica*), 15% by trematods (*Allocreadium isoporum, Diplostomum spathaceum*), 5% by nematods (*Contracoecum aduncum*) 5% by acanthocephalus (*Pomphorhynchus leavis*), 5% by protozoa (*Myxobolus carassi*), 5% by cestods (*Ligula intestinalis*).

CONCLUSIONS

From our investigations conducted in the research, we can say that there were no parasitic epizootic diseases that cause loss of fish species with economic value.

In Station 1, 14 species of parasites belonging to eight systematic groups were identified: Nematode, Monogenea, Trematode, Ciliata, Acanthocephala, Protozoa, Cestoda, Crustacea, while in Station 2, 11 species of parasites belonging to six systematic groups were identified: Monogenea, Ciliata, Trematoda, Nematoda, Acanthocephala, Cestoda.

The species *Sander lucioperca* and *Abramis brama* presented most often poly-parasitoses but with a low degree of infestation.

The most present group of parasites in Station 1 was represented by nematodes and in Station 2 by monogenic worms.

The presence of a relatively varied parasitosis, but with a low degree of infestation indicates a weaker effect of stressors, but to confirm these aspects we need future researchers.

ACKNOWLEDGEMENTS

The authors are grateful for the technical support offered by MADR Romania through the research project ADER 13.1.2/ 26.09.2019

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

HISTOPATHOLOGICAL CHANGES IN THE ALIMENTARY TRACT OF ALTUM ANGELFISH (*PTEROPHYLLUM ALTUM* PELLEGRIN, 1903) FED WITH MOSQUITO LARVAE (*CULEX* SPP.) IN HIGHLY ACIDIC WATER

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Abstract

Midge larvae, brine shrimp and Culex spp. larvae are important food items for many fin fishes, including Altum Angelfish. Chitin, the unbranched polymer present in the exoskeleton of arthropods, is not easily digestible by all fish species. Its edibility may also depend on variations of environmental factors to which fish are exposed, like acidic water. This is an unusual case of multifocal granulomatous pharyngitis and gastritis in Altum Angelfish, possibly caused by ingestion of mosquito (Culex spp.) larvae and exposure of the fish to highly acidic water (pH below 5.0). The microscopic examination of the larvae fed to the angelfish showed that the foreign bodies which caused the granulomatous reactions were exoskeleton residues of the Culex spp. larvae. However, the effect of acidic water on the integrity of the digestive tract of angelfish and the possible structural changes of the Culex larvae exoskeleton under these circumstances need to be demonstrated.

Key words*: Culex larvae, gastritis, pharyngitis, Pterophyllum altum, water pH.*

INTRODUCTION

Pterophyllum altum (Pellegrin, 1903), also known as Orinoco angelfish, deep angel or Altum Angelfish, is found in the wild in the upper Negro River drainage of the Amazon river basin and in tributaries of the upper Orinoco River (Inírida and Atabapo Rivers) to Puerto Ayacucho, in South America (Barreto-Reyes et al., 2015). These cichlids live in river watersheds where there are moderate amounts of water flow, submerged tree and plant roots and underwater vegetation where they can easily hide from predators. They are more frequently found in very soft and well oxygenated waters, and feed on insects, crustaceans, aquatic plants, small fish and food particles in the water.

Altums are the largest fish species in the Pterophyllum group, reaching 7 inches (18 cm) in length and about 9 inches (20 cm) in height from the tip of the dorsal fin to the tip of the anal fin. In the wild, there are, however, reports on specimens reaching twice the normal size.

The Altum Angelfish can be very challenging to keep in captivity. They are susceptible to stress related diseases, are sensitive to water quality fluctuations, and can be very difficult to breed and feed appropriately. Altums require food with high protein content, several feedings per day, and a strict water change regimen, of at least 25% every week. The water needs to be soft (2-5 dH) and slightly acidic (pH 5.5-6.5).

The paper presents a case of granulomatous pharyngitis and gastritis with intralesional chitinous foreign body in Altum Angelfish kept at a pH lower than 5, and fed food containing live mosquito larvae (*Culex* spp.). It is suspected that the low pH of the water compromised the mucosal integrity of the pharynx and stomach (Mota et al., 2018; Kennedy & Picard, 2012; Colt, 2006; Eshchar et al., 2006; Nagae et al., 2001; Allan & Maguire, 1992; Fromm, 1980) rendering the mucosae penetrable by the non-digestible filamentous chitin present in the exoskeleton of mosquito larvae.

MATERIALS AND METHODS

Eight adult Altum Angelfish were kept in three tanks for breeding purposes, and were fed a combination of midge fly larvae (*Chironomus circumdatus*, Kieffer), mosquito larvae (*Culex* spp.) and brine shrimp (*Artemia* spp.). Reverse

osmosis water is used, and water is recirculated through an external canister biofilter.

Seven months prior, the owner administered Tetra Paraguard™, as a preventive measure against parasites. This product is a combination of praziquantel, metronidazole, diflubenzuron and acriflavine. The owner mentioned that, during the time, the pH of the water was maintained at 6.0-7.5. Later, in an attempt to acidify the water, the owner added a mixture of peat (Fluval Peat Granules and Eheim TORF Pellets) in the external filter cannisters, and had made several water changes.

The veterinarian was called to examine the fish because some of them had not eaten for several days, were not breeding, and displayed neurological signs (depression and unresponsiveness). The water was tested with Sera ™ test kits for temperature, pH, total ammonia nitrogen (TAN), nitrite, nitrate and water hardness (KH). The values found are presented in the Table 1, below.

Table 1. Water quality parameters

	Units of measurement	Test results	Suggested optimal range
Temperature	°C	29.1	26-30
TAN	mg/L	5	0
Nitrite	mg/L	0	0
Nitrate	mg/L	0	0
pH	Units	5	5.5-6.5
KH	°dKH	≤ 1	2-4
GH	°dGH	0	2-5

Since it was assumed that the water pH had been fluctuating prior to the sampling, reaching levels below 5.0 due to the use of peat mixture without buffer addition, an experiment with the peat was conducted to determine how acidic it would make the water. In this experiment, Fluval Peat Granules and Eheim TORF Pellets were (separately) finely ground and soaked in Milli-Q® water and tap water, respectively (Figure 1). The samples were shaken for 2 minutes vigorously, left to stand for 5 minutes, and then incubated overnight at 4°C, 24°C and 37°C, respectively (Table 2).

Table 2. Water pH variations with two brands of peat added

Sample	Milli-Q® Water			Tap Water
Temperature	**4°C**	**24°C**	**37°C**	**24°C**
No peat (control)	-	5.64	-	6.84
Fluval	4.24	4.21	4.05	4.80
Eheim	3.99	3.95	3.95	4.12

The proportions used were: 1.0 g peat/30 ml Milli-Q® water, at an initial pH of 5 and, 1.0 g and 0.25 g peat/10 ml tap water, at an initial pH of 6. A set of controls (no peat added) with Milli-Q® water and tap water (24°C), with pH of 5.64 and 6.84, respectively, was used. Metrohm 691 pH meter was utilized to test the pH.

Figure 1. Fluval Peat Granules and Eheim TORF Pellets

The owner had preserved one deceased angelfish in the freezer, which was then held frozen for two days. After thawing, a partial necropsy was conducted, to allow better perfusion for fixation in 10% neutral-buffered formalin. The fish was held in fixative for 24 hours, and then decalcified for 3 hours in a formic acid solution. Tissues were then routinely processed into paraffin blocks, from which 4 µm sections were cut and stained with haematoxylin and eosin. The skin, muscle, gills, eye, pharynx, heart, spleen, kidney, liver, stomach, intestines and fin were examined using routine light microscopy. Wet preparations of the live food used to feed the fish were also analysed by light microscopy, after fixation in 10% neutral-buffered formalin.

RESULTS AND DISCUSSIONS

1. The peat experiment showed a reduction in the pH below tolerance range in MilliQ® water and tap water (Table 2). The water pH varied with the type of water used in the sample (*i.e.*, MilliQ and tap water), water temperature, and the type of peat used in the experiment. Peat added to Milli-Q® water at 24°C reduced the pH from 5.64 (in controls) to pH 3.95, in the Eheim peat. In tap water samples (initial pH 6.84, in controls) the peat decreased the pH to 4.12 in Eheim peat, and 4.80 in Fluval peat. A severe reduction of pH was recorded in tap water samples (initial pH 6.84) rather than in

Milli-Q® water samples (initial pH 5.64), with 2.72 units (Eheim peat) and 2.04 units (Fluval peat), respectively, versus 1.69 units (Eheim peat) and 1.43 units (Fluval peat), respectively.

2. Histopathological findings in the fish samples. Significant findings were found in the pharinx and stomach of the examined fish. In the proximity of the pharyngeal teeth, in the pharinx mucosa and the mucous glands of the pharinx, there were diffuse granulomatous reactions surrounding golden-brown, refractile, structures. The structures were cilindrical with variable diameters on cross section, or had parallel-sided walls. These bodies were often observed occuring in clusters (Figure 2 and Figure 3).

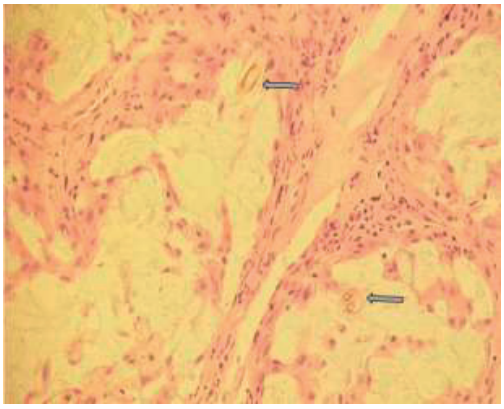

Figure 2. Foreign body granuloma in the mucous glands of the pharynx (arrows). Histological section, H E, 20x

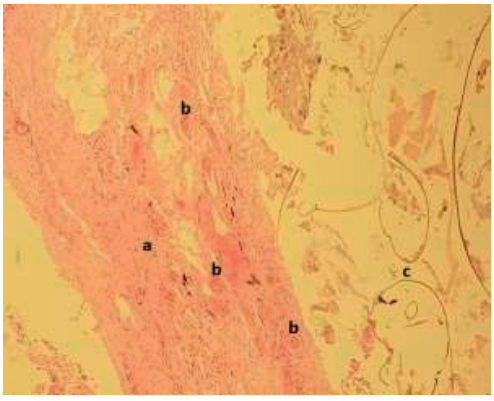

Figure 3. Foreign body granulomatous inflammation in the stomach wall. (a) Hyperplasia. (b) Granulomatous gastritis with clusters of chitin residues. (c) Chitin filaments in the gastric lumen. Histological section, H E, 10x

In the stomach wall, there were similar multifocal granulomatous reactions centred around tube-like, golden-brown structures occuring in bundles or singly, in longitudinal, cross and tangential sections (Figure 3). In the lumen of the stomach there were filaments and fragments of similar material found in the mucosa (Figure 4).

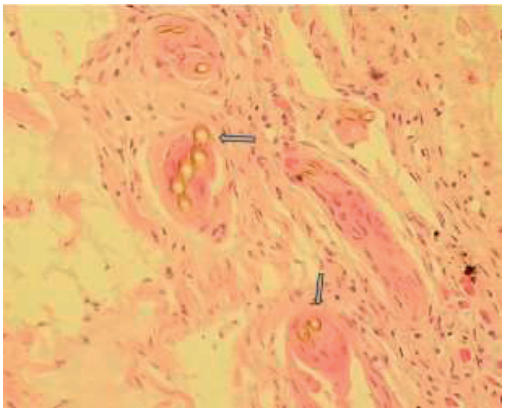

Figure 4. Chitinous foreign body granuloma (arrows) in the gastric epithelium. Histological section, H E, 40x

3. The live food components description: In light microscopy, the body of midge fly larva was as thin, cylindrical and segmented. The posterior end of the body featured short hair-like chitinous filaments (Figure 5).

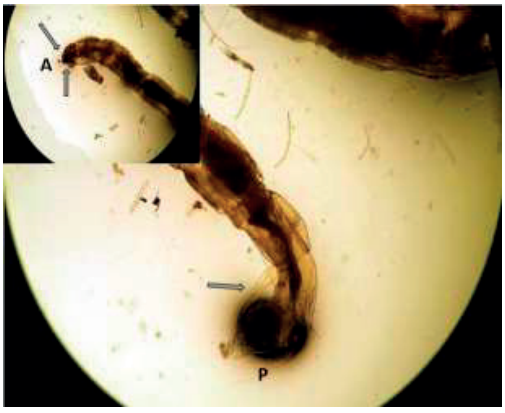

Figure 5. Midge larva external structure. Anterior (A) and posterior (P) ends feature parapods and procerci (arrows)

The brine shrimp exoskeleton appeared thin, covered by short filaments of chitin on the thorax and posterior end of the body (Figure 6).

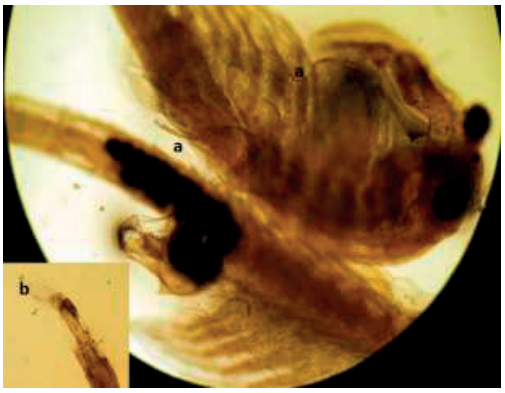

Figure 6. Brine shrimp exoskeleton. (a) Chitinous filaments on torax. (b) Chitinous hairs on the tail

The mosquito larva body had numerous tufts of long hair-like structures on the head, thorax and abdomen. Additionally, there were abundant anal and dorsal brushes and hairs on the posterior end of the body (Figure 7), resembling the structures observed in the pharinx and stomach of the angelfish.

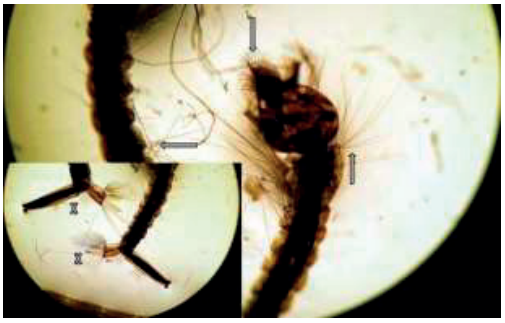

Figure 7. *Culex* spp. larvae exoscheleton. Strands of hairs on the head, thorax and abdomen (arrows). Abundant anal brushes and caudal hairs on the posterior end of the body (X)

Dry commercial, freeze dried or frozen foods often cannot meet all the nutritional requirements for conditioning broodstock, hence supplementation of their diet with live food is common practice (Farahi et al., 2010). Like in the case of *Artemia* (brine shrimp), adult insects and their larvae are largely utilized in fish rearing, being part of the natural diet of fish (Howe et al., 2014; Whitley and Bollens, 2014; Henry et al., 2015). Midge larvae, brine shrimp and *Culex* spp. larvae are recognized as important food items for many ornamental fishes (Patra & Ghosh, 2015). Insects and their larvae are rich in protein, fats, vitamins and minerals (Zamprogna et al., 2017). Artemia has a high nutritive value and conversion efficiency (Kaiser et al., 2003; Farahi et al., 2010). The unbranched polymer chitin is the primary component of the exoskeleton of arthropods (crustacean shells, insect exoskeletons), (Henry et al., 2015). Chitin digestibility varies amongst different fish species due to the variety of chitinase activity (Zamprogna et al., 2017). However, it is highly unusual for finfish to experience the injuries observed in this case from eating the arthropod juveniles and larvae. Few species of mosquito larvae survive below pH 4.0 (Thamer and Abdulsamad, 2005; Armesto et al., 2017; Clark et al., 2004). We are hypothesizing that the pH of water must have played an important role in the development of the lesions observed in the alimentary tract of the Angelfish fed the live food.

CONCLUSIONS

Following microscopic examination of the feed, it was concluded that the most likely candidate to have caused the lesions seen in the pharynx and stomach was the mosquito larvae, due to its abundant hairs of chitin. It is suspected that the low pH of the water compromised the mucosal integrity of the pharynx and stomach rendering the mucosae penetrable by the non-digestible filamentous chitin present in the exoskeleton of mosquito larvae. It is also possible that the acidic water to have altered the exoskeleton structure of the mosquito larvae, rendering it indigestible to the altum fish. The peat experiment showed that the peat used to correct water pH in the altums tanks may have reduced the pH below the tolerance range. The water could have, at some time, decreased to a pH lower than 4.0, as showed by the peat experiment results. In addition, the pH falling below 5.0 likely resulted in harming the microorganisms in the biofilter, explaining the elevated ammonia. Though, this level of ammonia is unlikely to have caused toxicosis since there would be negligible free-ammonia nitrogen at this low pH. While it is recognizable that the foreign bodies which caused the granulomatous reactions in the stomach and pharynx of the

Angelfish are residues of the exoskeleton of, mainly, the mosquito larvae, the effects of acidic water on the integrity of digestive tract in Angelfish fed live food needs to be further documented. Whether the acidic water may have affected the structure of the exoskeleton, rendering it indigestible by the fish, thus causing the foreign body reactions in the stomach and pharynx, this also needs to be documented further.

ACKNOWLEDGEMENTS

The authors thank Mai Nguyen for help with testing the acid-effect of peat.

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

PARASITES AND PARASITE COMMUNITIES OF *VIMBA VIMBA* (LINNAEUS, 1758) FROM THE DANUBE RIVER, NORTHWESTERN BULGARIA

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Abstract

In 2020, 37 specimens of vimba bream (Vimba vimba Linnaeus, 1758) from the Danube River near the village of Kudelin were examined for parasites. Four parasite species were established: Nicolla skrjabini (Iwanitzky, 1928) and Posthodiplostomum cuticola (von Nordmann, 1832) (Trematoda); Pomphorhynchus laevis (Zoega in Müller, 1776) (Acanthocephala) and Philometra rischta Skrjabin, 1917 (Nematoda). The study aims to provide new data on parasites and parasite communities of Vimba vimba from the Danube River's freshwater ecosystem near the village of Kudelin in northwestern Bulgaria. In the study, the prevalence (P%), mean intensity (MI), mean abundance (MA) and the Brillouin's diversity index (HB) were presented and discussed.

Key words*: Bulgaria, Danube River, parasites, parasite communities, Vimba vimba.*

INTRODUCTION

The Danube River is one of the largest rivers in Europe, with a length of 2,857 km (Ilie et al., 2017). The river passes through many countries, while for others, the river only forms its borders (Pantelica et al., 2012).

The Danube River is the border between the Republic of Bulgaria and the Republic of Romania in 470 km - a sector characterized by a great fish diversity (Zarev et al., 2013).

The fish are hosts of various parasite species (Amer, 2014). Not only the ichthyofauna of the Danube River but also the parasite fauna of different fish species from the Bulgarian section of the river is a subject of research by several authors (Chunchukova & Kirin, 2017; Chunchukova et al., 2017; Chunchukova & Kirin, 2018; Chunchukova et al., 2018; Chunchukova & Kirin, 2020; Chunchukova et al., 2020; Zaharieva & Kirin, 2020a; 2020b; Zaharieva & Zaharieva, 2020a; 2020b; 2020c; 2020d). Research on parasites of *Vimba vimba* (Linnaeus, 1758) from the Danube River and its basin was conducted by a few authors (Diaconescu et al., 2010; Djikanović et al., 2012).

The present study aims to provide new data on parasites and parasite communities of vimba bream from the Danube River, Kudelin village, northwestern Bulgaria.

MATERIALS AND METHODS

In 2020, 37 specimens of *Vimba vimba* (Linnaeus, 1758) from the upper current of the Bulgarian section of the Danube River in the Kudelin village's vicinities were caught and studied. The village of Kudelin (44°11′30″N, 22°40′5″E) is located in the Vidin Lowland, in the North-Western part of Bulgaria. The village is situated in the immediate proximity of the borders between three countries - Bulgaria, Romania and Serbia (Figure 1).

Figure 1. Danube River, Kudelin village, Bulgaria (www.icpdr.org)

Using fishing gear specified in a fishing permit were caught the fish. The permit was issued by the Executive Agency of Fisheries and Aquaculture (EAFA). According to Karapetkova & Zhivkov (2006) were identified the caught fish. The weight (G), as well as the maximum length (L) and maximum height (H) of the body of the studied specimens *V. vimba*, were recorded (Table 1).

Table 1. Maximum body length, height and weight (L, H and G) of *Vimba vimba* from the Danube River (Kudelin village)

Vimba vimba **(N = 37)**	**Min. - max.**	**Mean ± SD**
L (cm)	15.5-33	20.68 ± 5.90
H (cm)	3.3-8.5	4.87 ± 1.55
G (g)	32-486	111.24 ± 116.14

All 37 collected specimens of vimba bream were tested for parasites according to methods described by Petrochenko (1956); Zashev & Margaritov (1966); Kakacheva-Avramova (1983); Bauer (Ed.) (1987); Moravec (2013). Permanent and temporary microscopic slides were prepared, according to Zashev & Margaritov (1966); Georgiev et al. (1986), and Moravec (2013), to identify the parasite species. In this study, the prevalence (P%); mean intensity (MI); mean abundance (MA) and Brillouin's diversity index (HB) were calculated and presented (Magurran, 1988; Bush et al.,1997).

RESULTS AND DISCUSSIONS

The subject of this parasitological research were 37 specimens of vimba bream (*Vimba vimba* Linnaeus, 1758). The fish were caught in 2020 from the Danube River near Kudelin, located in the northwestern part of Bulgaria. *Vimba vimba* is a freshwater, brackish, benthopelagic fish from the Cyprinidae family (Karapetkova & Zhivkov, 2006; Froese & Pauly, 2020).

Helminth community structure

Parasitological examination of vimba bream *V. vimba* from the Danube River (Kudelin) revealed the presence of four species of parasites: two parasite species of class Trematoda: *Nicolla skrjabini* (Iwanitzky, 1928) and *Posthodiplostomum cuticola* (von Nordmann, 1832); one parasite species of class Acanthocephala: *Pomphorhynchus laevis* (Zoega in Müller, 1776) and one parasite species of class Nematoda: *Philometra rischta* (Skrjabin, 1917) (Table 2).

Table 2. Parasite species diversity of *Vimba vimba* from the Danube River, Kudelin

Parasite species	***Vimba vimba*, Danube River, Kudelin, 2020**
Nicolla skrjabini (Iwanitzky, 1928)	•
Posthodiplostomum cuticola (von Nordmann, 1832), metacercaria	•
Pomphorhynchus laevis (Zoega in Müller, 1776)	•
Philometra rischta (Skrjabin, 1917)	•

Component community

The component community of *Vimba vimba* from the Danube River, Kudelin, northwestern Bulgaria, was studied. The trematodes were present at the highest number (2 species with > 1,205 specimens), followed by the acanthocephalans (1 species with 99 specimens). The nematodes had the smallest number of specimens (1 species with four specimens). In the component community of

V. vimba from the Danube River, Kudelin, *Posthodiplostomum cuticola*, and *Pomphorhynchus laevis* were core parasite species with a prevalence (P%) respectively P% = 32.43 and P% = 29.73. *Philometra rischta* (P% = 8.11) and *Nicolla skrjabini* (P% = 5.41) were accidental parasite species in the parasite community of vimba bream. The highest mean intensity (MI) and the highest mean abundance (MA) were found for the parasite *P. cuticola* (MI = 100.00; MA = 32.43) (Table 3).

Table 3. Main ecological terms of parasite and parasite communities of *Vimba vimba* from the Danube River, Kudelin

Parasite species	Kudelin N = 37					
	n	p	MI	MA	P%	Range
Nicolla skrjabini (Iwanitzky, 1928)	2	5	2.50	0.14	5.41	1-4
Posthodiplostomum cuticola (von Nordmann, 1832), metacercaria	12	> 1,200	100.00	32.43	32.43	> 100
Pomphorhynchus laevis (Zoega in Müller, 1776)	11	99	9.00	2.68	29.73	1-47
Philometra rischta Skrjabin, 1917	3	4	1.33	0.11	8.11	1-2

N - number of investigated fish, n - number of infected fish, p - number of fish parasites, MI - mean intensity, MA - mean abundance, P% - prevalence.

Infracommunity

Of the thirty-seven specimens of vimba bream subjected to parasitological examination, it was found that 13 specimens of *V. vimba* or 35.14% were not infected; 20 specimens of *V. vimba* or 54.05% were infected with one parasite species, and four specimens of *V. vimba* or 10.81% were infected with two parasite species (Figure 2; Table 4).

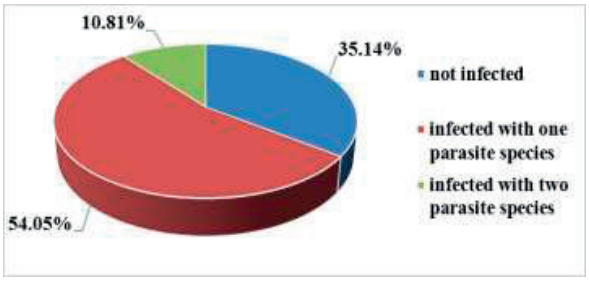

Figure 2. Infection of *Vimba vimba* from the Danube River, Kudelin

The study showed that in the parasite infracommunity of vimba bream, the number of detected parasites ranged from 1 to > 100. The subject of research was more than 1,308 specimens of parasites. Brillouin's diversity index is low (Table 4).

Table 4. Infracommunity of *Vimba vimba* from the Danube River, Kudelin

Number of specimens *Vimba vimba*	Number of parasite species		
	0	1	2
	13	20	4
Total number of species (Mean number of species ± SD)	4 (0.18 ± 0.36)		
Total number of specimens (Mean number of specimens ± SD)	> 1.308 (8.84 ± 14.15)		
Brillouin's diversity index (HB)	0.308		

Few authors have carried out studies on parasites of *Vimba vimba* (Linnaeus, 1758) from the Danube River and rivers in the Danube basin. For the Bulgarian section of the river, the species *N. skrjabini* has been reported for the region of the city of Vidin (Koshava village; Novo selo village; Vetren village, etc.). *V. vimba* is a new host record for *N. skrjabini*. Kudelin is a new locality of the Danube Water Basin for the parasite species. *N. skrjabini* has been reported for other species of freshwater fish (Margaritov, 1959; Kakacheva-Avramova, 1977; Kakacheva et al., 1978; Atanasov, 2012; Kirin et al., 2013; Zaharieva & Kirin, 2020a). *P. citicola* was reported for *Alburnus alburnus* near the villages of Kudelin and Novo Selo (Zaharieva & Kirin, 2020a). The species *P. laevis* was known for the Bulgarian section of the Danube River of the host *V. vimba*, from the region of Vidin, but is not reported for Kudelin (Kakacheva-Avramova, 1977; Kakacheva et al., 1978), and also as a parasite of *Alburnus alburnus* and *Chondrostoma nasus* from Kudelin (Zaharieva & Kirin, 2020a; 2020b). The nematode *Ph. rischta* has not been reported for the parasite fauna of *V. vimba* in Bulgaria. The species is reported for the first time for the Bulgarian section of the Danube River. Zaharieva & Zaharieva (2020c; 2020d) found 6 and 5 parasite species of *Abramis brama* from the Danube River (Kudelin), including *N. skrjabini*, *P. cuticola* and *P. laevis*. Nedeva et al. (2003) reported *P. laevis* in the Danube River in the Republic of Serbia and the Bulgarian sector of the river (the villages Archar, Botevo, Gomotartsi), including on host *V. vimba*. Leimgruber et al. (2005) reported *P. laevis* in the Austrian section of the Danube River. The species was announced in the Danube River's Czech Republic and Slovakia sections (Moravec, 2001). Diaconescu et al. (2010) studied 11 fish species for parasites from the Danube River Delta in Romania. The authors found the trematode *Posthodiplostomum cuticola* on *V. vimba*, etc.

CONCLUSIONS

In 2020, 37 specimens of *Vimba vimba* (Linnaeus, 1758) from the Bulgarian section of the Danube River close to the village of Kudelin were caught and subjected to ecoparasitological studies. Twenty four specimens of *V. vimba* were infected with four parasite species – the trematodes *N. skrjabini* and *P. cuticola*, the acanthocephalans *P. laevis* and the nematodes *Ph. rischta*. Two species of parasites *P. cuticola* (P% = 32.43) and *P. laevis* (P% = 29.73) were core parasite species in the component community of *V. vimba*. The Brillouin's diversity index was low (HB = 0.308) due to the presence of only four species and the apparent dominance of one species with a very high number (*P. cuticola*). *V. vimba* is a new host for *N. skrjabini*, *P. cuticola*, *Ph. rischta* in Bulgaria. *Ph. rischta* is reported for the first time for the Bulgarian section of the Danube River. The Danube River, Kudelin village, is a new habitat for *Ph. rischta* as parasites of *V. vimba* from this study.

ACKNOWLEDGEMENTS

We are grateful to the Agricultural University – Plovdiv and the Centre of Research, Technology Transfer and Protection of Intellectual Property Rights for the approved funding for project No. 05-20, "Support of doctoral programs".

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Scientific Papers. Series D. Animal Science. Vol. LXIV, No. 1, 2021
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

CIRCULATION OF CADMIUM (CD) IN THE SYSTEM *ALBURNUS ALBURNUS* (LINNAEUS, 1758), WATER AND SEDIMENTS FROM THE DANUBE RIVER, NORTHWESTERN BULGARIA

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Abstract

In 2020, 30 specimens of bleak (Alburnus alburnus Linnaeus, 1758), 3 samples of water and 3 samples of sediments were collected from the Danube River (Kudelin) in the northwestern part of the Republic of Bulgaria. Samples of water and sediments, of tissues/ organs (liver, skin and muscles) of bleak were investigated for the presence of cadmium (Cd). The concentrations of Cd in the studied tissues/ organs of Alburnus alburnus decreased as follows: liver (C_{Cd} = 1.38 ± 0.55 mg.kg^{-1} wet weight) > skin (C_{Cd} = 0.50 ± 0.18 mg.kg^{-1} wet weight) > muscles (C_{Cd} = 0.09 ± 0.05 mg.kg^{-1} wet weight). The reported concentrations of Cd in water samples are C_{Cd} = 0.008 ± 0.006 mg.l^{-1}, and in sediments samples C_{Cd} = 1.55 ± 2.35 mg.kg^{-1} dry weight.

Key words*: Alburnus alburnus, cadmium, Danube River, Kudelin, northwestern Bulgaria.*

INTRODUCTION

With a length of 2,857 km, the Danube River ranks second in length in Europe (Baltălungă & Dumitrescu, 2008). The river has hundreds of tributaries - approximately 300 (Gasparotti et al., 2013).

Larger tributaries of the Danube River are the Morava River with a length of nearly 270 km (the Czech Republic/Slovakia/ Austria), the Tisza River with a length of 977 km (Ukraine/Slovakia/Romania/Hungary/ Serbia), the Sava River approximately 944 km (Slovenia/Croatia/Bosnia and Herzegovina/ Serbia), Timok River with a length of 202 km (Serbia/ Bulgaria), Iskar River - 368 km (Bulgaria), Olt River with a length of 615 km (Romania), Prut River - 967 km (Romania/ Moldova), etc. (Parvanov et al., 2008; Postolachi et al., 2012; Sakan et al., 2013; Serbula et al., 2016; Kilianova et al., 2017; Iordache et al., 2019; Bakiu, 2020).

The Danube River receives water from its numerous tributaries and is highly vulnerable to contamination (Frincu et al., 2020).

Heavy metals are among the most hazardous pollutants in the aquatic environment due to their toxicity and accumulation in aquatic organisms and the surrounding environment (Janjić et al., 2015). In fish, cadmium accumulates chiefly in the kidneys and liver (Kumar & Singh, 2010). There are few studies on heavy metals content in tissues/ organs of bleak from the Bulgarian section of the Danube River (Chunchukova & Kuzmanova, 2017; Chunchukova et al., 2017; Shukerova et al., 2017; Chunchukova, 2018; Chunchukova et al., 2020; Zaharieva & Kirin, 2020).

The study aims to provide information on the circulation of cadmium (Cd) in the system bleak (*Alburnus alburnus* Linnaeus, 1758), water and sediments from the Bulgarian section of the Danube River, near the village of Kudelin, northwestern Bulgaria.

MATERIALS AND METHODS

Samples of water and sediments, and fish, during 2020 were collected from the Danube River, close to Kudelin. Kudelin (44°11′30″N, 22°40′5″E) is a village in northwestern Bulgaria. It is located in Vidin district and is situated near the Timok River and Danube River, which form part of the border of Bulgaria with the Republic of Serbia and Republic of Romania (Figure 1).

Figure 1. Danube River, Kudelin, northwestern Bulgaria (www.icpdr.org)

The scientific fishing was carried out using fishing gear mentioned in a permit issued by the Executive Agency of Fisheries and Aquaculture (EAFA). The species of all caught fish was defined by Karapetkova & Zhivkov (2006). TL, MH and BW namely the total length (cm), the maximum body height (cm) and the body weight (g) of the studied specimens of *A. alburnus*, were noted (Table 1).

Table 1. Length (TL), height (MH) and weight (BW) of the studied specimens of *A. alburnus* from the Danube River, Kudelin

***Alburnus alburnus* N = 30**	**TL**	**MH**	**BW**
Min – Max	9.1-12.5	1.7-2.9	4-12
Mean ± SD	10.61 ± 0.86	2.26 ± 0.27	6.40 ± 1.69

Three samples of water, three samples of sediments, as well as tissues and organs samples (liver, skin and muscles) of bleak, were sent for chemical analysis on ICP "OPTIMA 7000" Perkin-Elmer in an accredited laboratory at the Institute of Biodiversity and Ecosystems Research at the Bulgarian Academy of Sciences, Sofia, Bulgaria. In the study was fixed the bioconcentration factor (BCF). The linear correlation coefficient of Spearman (r_s) was also calculated.

RESULTS AND DISCUSSIONS

In 2020, 30 specimens of *Alburnus alburnus* (L., 1758) from the Danube River's freshwater ecosystem, Kudelin, were studied. *Alburnus alburnus* is a freshwater fish that inhabits the upper layers of the water. The species is subject to sport fishing (Karapetkova & Zhivkov, 2006).

Liver, skin and muscles samples of *A. alburnus* were analyzed for cadmium presence (Cd). The chemical analysis data are given in $mg.kg^{-1}$ wet weight; $mg.kg^{-1}$ dry weight. Water samples and sediments samples from the same section of the river were also collected and tested for Cd content. The chemical analysis data of water samples are in $mg.l^{-1}$ and of sediments samples in $mg.kg^{-1}$ dry weight (Table 2).

Table 2. Cadmium (Cd) concentrations in tissues/ organs of *A. alburnus*, water and sediments from the Danube River, Kudelin

Tissues/organs of *A. alburnus*, water, sediments		**Min - Max**	**Mean ± SD**
liver	$mg.kg^{-1}$ wet weight	0.78-1.86	1.38 ± 0.55
	$mg.kg^{-1}$ dry weight	2.27-6.79	4.25 ± 2.31
skin	$mg.kg^{-1}$ wet weight	0.38-0.70	0.50 ± 0.18
	$mg.kg^{-1}$ dry weight	0.48-0.84	0.61 ± 0.21
muscles	$mg.kg^{-1}$ wet weight	0.05-0.15	0.09 ± 0.05
	$mg.kg^{-1}$ dry weight	0.13-0.37	0.23 ± 0.12
water	$mg.l^{-1}$	0.001-0.011	0.008 ± 0.006
sediments	$mg.kg^{-1}$ dry weight	0.15-4.27	1.55 ± 2.35

Of the examined tissues/ organs of *A. alburnus*, the highest concentrations of Cd were reported in the liver samples (C_{Cd} = 1.38 ± 0.55 mg.kg^{-1} wet weight), followed by those in skin samples (C_{Cd} = 0.50 ± 0.18 mg.kg^{-1} wet weight) and muscles samples (C_{Cd} = 0.09 ± 0.05 mg.kg^{-1} wet weight). The study showed that the examined element concentrations in samples of bleak decreased in the order: liver > skin > muscles. During the study, concentrations of Cd in the water samples (C_{Cd} = 0.008 ± 0.006 mg.l^{-1}) and in the sediments samples (C_{Cd} = 1.55 ± 2.35 mg.kg^{-1} dry weight) from the Danube River, Kudelin were also indicated (Table 2).

The cadmium (Cd) concentrations in the liver, skin and muscles of bleak were studied in 2019 by Zaharieva & Kirin (2020) from the same section of the Danube River. The authors found that concentrations of cadmium in bleak samples decreased in the order: liver (C_{Cd} = 0.80 ± 0.56 mg.kg^{-1} wet weight) > skin (C_{Cd} = 0.21 ± 0.15 mg.kg^{-1} wet weight) > muscles (C_{Cd} = 0.08 ± 0.07 mg.kg^{-1} wet weight), which was confirmed in the present study. In 2020, higher Cd concentrations were reported in the liver, skin and muscles samples of *A. alburnus* compared to those found in 2019, by 1.73 times, 2.38 times and 1.13 times, respectively.

The study presented cadmium (Cd) excess in tissues/ organs samples of *A. alburnus* in relation to the norms specified in national and international documents. The Cd concentrations in liver, skin and muscles of *A. alburnus* exceeded the standard for Cd (0.05 mg/kg) in Ordinance No. 31 of 2004 on the maximum levels of contaminants in foodstuffs, by 27.6, 10 and 1.8 times, respectively. The reported Cd concentrations in the liver and skin of *A. alburnus* exceeded the norm (0.2 mg/kg) given by the Food and Agriculture Organization (FAO) by 6.9 and 2.5 times, respectively (Figure 2).

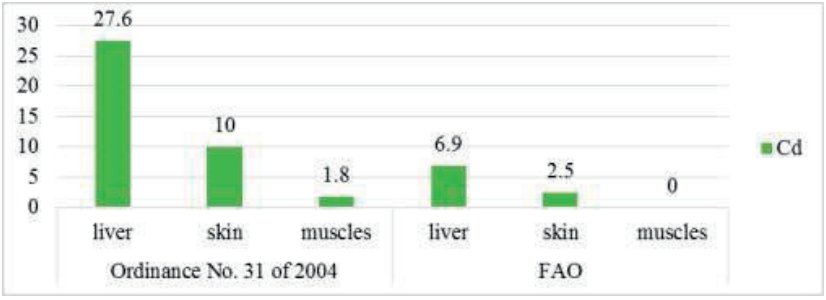

Figure 2. Excess of Cd in tissues/ organs (mg.kg^{-1} wet weight) of *A. alburnus* from the Danube River, Kudelin

The concentrations of cadmium (Cd) in water samples were considered with the norms in documents from the national legislation (Ordinance on environmental quality standards for priority substances and certain other pollutants of 2010; Ordinance No. 18 of 2009 on the quality of water for irrigation of crops). Excess of Cd in water was found only with the norm (0.0009 mg/l) in the Ordinance on environmental quality standards of 2010, namely by 8.89 times (Figure 3).

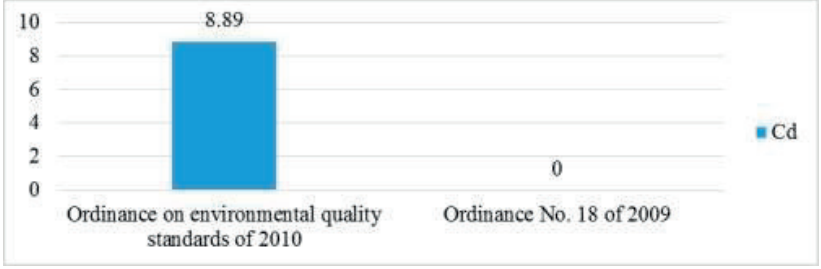

Figure 3. Excess of Cd in water (mg/l) from the Danube River, Kudelin

The study compared the cadmium concentrations (Cd) in sediment samples with the norms specified in national and international documents. The Cd concentrations in sediments did not exceed the norm (2 mg/kg at pH 6.0-7.4) in Ordinance No. 3 of 2008 on the norms for the permissible content of harmful substances in soils. Still, they exceeded the Dutch Target Values (0.8 mg/kg) by 1.94 times (Figure 4).

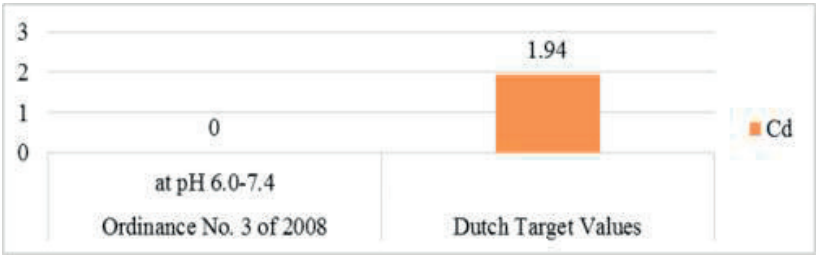

Figure 4. Excess of Cd in sediments (mg/kg) from the Danube River, Kudelin

The bioconcentration factor of water ((BCF = [$C_{host\ tissues}$]/[C_{water}])) and the bioconcentration factor of sediments ((BCF = [$C_{host\ tissues}$]/[$C_{sediments}$])) were calculated. In both cases, the liver samples had the highest Cd accumulation, and the muscles samples had the lowest (Table 3).

Table 3. Bioconcentration factor BCF and BCF

***Alburnus alburnus* /Water**	**BCF_{Cd}**
C_{liver}/C_{water}	172.50
C_{skin}/C_{water}	62.50
C_{muscle}/C_{water}	11.25
***Alburnus alburnus*/Sediments**	**BCF_{Cd}**
$C_{liver}/C_{sediments}$	2.74
$C_{skin}/C_{sediments}$	0.39
$C_{muscle}/C_{sediments}$	0.15

The linear correlation coefficient of Spearman (r_s-1.0) shows very high correlations between the Cd content in water and sediments and those in the studied liver, skin and muscles samples of *A. alburnus*.

Chunchukova & Kuzmanova (2017) found a significant negative correlation ($p<0.05$) between concentrations of arsenic in *Pomphorhynchus laevis* (acanthocephalan on *A. alburnus*) and those in sediments from the Danube River (Vetren). Chunchukova et al. (2017) fixed Spearman's rank correlation coefficient (r_s). They found a highly significant correlation ($p<0.01$) between the concentrations of lead (Pb) in *Pomphorhynchus tereticollis* (acanthocephalan on *A. alburnus*) and those in the Danube River's sediments (the Vetren area). Chunchukova (2018) reported a significant correlation ($p<0.05$) between the nickel (Ni) content in *P. laevis* and those in skin samples of bleak from the Danube River (Vetren). Zaharieva & Kirin (2020) found a very high correlation between the content of copper, cadmium and arsenic in tissues/ organs of *A. alburnus* and those in Danube River's water and sediments (the Kudelin biotope) – r_s=0.86-0.99 relative to water content; $p<0.05$ and r_s=0.96-0.99; $p<0.05$.

CONCLUSIONS

In 2020, 30 specimens of *A. alburnus* (L., 1758), three water samples, and three samples of sediments were collected from the Danube River (Kudelin). All collected samples were tested for the presence of cadmium (Cd). With regard to the examined tissues/ organs of bleak, cadmium concentrations were the highest in the liver samples (C_{Cd} = 1.38 ± 0.55mg.kg^{-1} wet weight), followed by those in

the skin samples (C_{Cd} = 0.50 ± 0.18 mg.kg^{-1} wet weight) and the muscles samples (C_{Cd} = 0.09 ± 0.05 mg.kg^{-1} wet weight). The bioconcentration factor values prove the highest Cd accumulation occurred in the liver and the lowest in muscles for both the water and the sediments. A very significant correlation was found between the Cd content in both water and sediments, and those in the bleak studied biological samples.

ACKNOWLEDGEMENTS

The chemical analyzes were performed in an accredited laboratory at the Institute of Biodiversity and Ecosystem Research at the BAS, Sofia, Bulgaria, for which we are grateful. We also thank the Centre of Research, Technology Transfer and Protection of Intellectual Property Rights at the Agricultural University – Plovdiv for the funds received under project No. 04-20 in the direction of "Support of doctoral programs", which helped to conduct chemical analysis.

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ISSN 2285 – 5750
ISSN-L 2285 – 5750