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EFFECT OF COOLING AND FREEZING ON THE KINEMATIC PARAMETERS OF RAM SPERMATOZOA SEXED BY MODIFIED PROTOCOL WITH TLR7/8 LIGAND R848

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Abstract: The present study investigated the effect of sex-sorting by TLR7/8 ligand R848, cooling and cryopreservation on the motility and kinematic characteristics of ram spermatozoa for the first time. Four ejaculates per ram (n=2) were collected, pooled and after that were split to 8 equal parts. Four parts were used for sexing and four parts were analyzed as unsexed semen. The sperm sexing was done by application of the TLR7/8 ligand R848 (resiquimod). Diluted with freezing extender unsexed and sex-sorted semen were cooled in a refrigerator at temperature 5°C for 5 hours and after that freeze/thawed. Each part of semen at each step of the experiment was subjected to computer assisted semen analysis for evaluation of motility and speed parameters of spermatozoa. After sexing the significant reducing of kinematic parameters in both fractions were observed. The dilution of sexed spermatozoa with freezing extender before cooling reliably increased the progressive motility and speed parameters as VCL, VAP, and VSL in X- and Y-fractions and kept them during the cooling at relatively high level. Freezing adversely impaired the motility of sexed spermatozoa. The values of speed parameters of unsexed spermatozoa after thawing were significantly higher than those of X and Y spermatozoa. Dilution with an appropriate freezing extender and cooling are most suitable approaches for storing of sexed ram spermatozoa.

Key words: ram semen sexing, TLR7/8 ligand R848, kinematics, cooling, freezing

Introduction

The introduction of sex-sorted sperm for AI in small ruminants is of great importance for increasing the reproductive efficiency, utilizing the genetic resources, and producing the optimal sex-ratio of offspring in production systems (Gonzalez-Marín et al., 2021). Sperm sexing by flow cytometry is considered as an accurate and effective method for offspring sex selection (Vishwanath and Moreno, 2018). This pre-selection of spermatozoa bases on the different DNA amount between the X and Y chromosomes (Johnson, 2000). Recently, the flow cytometric procedure for sorting of ram semen was also commercialized (Gonzalez-Marín et al., 2021). However, the currently available commercial method still presents many limitations like high cost, low sperm number per dose, sperm damage and decreased fertility (Quelhas et al., 2021). Additionally, the use of this technology requires expensive equipment and skilled personnel. Regardless the fact that at this stage only flow cytometry gives a separation with an accuracy of up to 90%, the scientific search for different ways for sexing of ram sperm are still in progress.

The main goal of these studies is to discover an easier method for semen sexing, maintaining good quality of sperm after treatment and to make it accessible to animal husbandry practice. Two methods, adapted to these requirements have been recently tested for ram spermatozoa. One of them uses bovine serum albumin (BSA) gradient for spermatozoa separation (Hadi and Al-Timimi, 2013; Solihati et al., 2019a; Yotov et al., 2021). Another is based on the activation of *Toll- like receptor 7/8 (TLR7/8)* on X- sperm by ligand resiquimod (R848) (Umehara et al., 2020; Ren et al., 2021; Abadjieva et al., 2022; Yotov et al., 2024a). Necessity to inseminate a large flock of ewes after estrus synchronization for 1-2 days provoke the investigations about the appropriate way for storage of the sexing spermatozoa fractions. While there are some data about the effect of cooling and freezing on the motility and survivability of ram spermatozoa sexed by BSA column (Maxwell et al., 1984; Solihati et al., 2019b; Yotov et al., 2024b), information on storage of ram spermatozoa separated by R848 in cooled and frozen state is not available.

The objective of present study was to investigate the possibility of storage of ram spermatozoa, sexed by modified R848 protocol, by cooling and freezing. Given the objective, the effects of freezing extender and the cooling and freezing processes on the motility and different kinematic parameters of non-sexed and sexed ram spermatozoa were analyzed and compared.

Material and Methods

Animals, semen collection, primary assessment and processing

The investigation was performed with four ejaculates per Pleven Blackhead ram (n=2) collected by artificial vagina method. After semen collection

the samples were transported to the laboratory, placed on a water bath at 37°C, and submitted to a primary assessment.

Only ejaculates with normal colour and transparency, volume > 1.5 mL, sperm concentration > 1.5 $\times 10^9$/mL, motile sperm > 70% and lack of agglutination were used in this experiment. To minimize ram individual effect all the ejaculates were pooled and after that were split to 8 equal parts. Four parts were used for sexing and four parts were analyzed as non-sexed semen. Each part of the semen was subjected to computer assisted semen analysis (CASA, Microptic S.L. Barcelona, Spain) after each step of the procedures. The sperm parameters (motility characteristics and average values of speed) were evaluated by three measurements of each part used for sexing and non-sexing by analyzing six areas per samples. The mean values were considered as final.

The motility characteristics included progressive motility (PR, %), non-progressive motility (NP, %) of spermatozoa, immotile sperm (IM, %), total motility (TM, %) velocity (rapid - R and medium - M; %) and velocity and progressivity (slow - S, rapid - RP and medium - MP; %). The registered values of speed of motile spermatozoa were curve linear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s) average path velocity (VAP, μm/s), linearity index (LIN, %), straightness (STR, %) and oscillation index (WOB, %). Additionally, amplitude of lateral head movement (ALH, μm), beat frequency (BCF, Hz) and percent of hyperactive spermatozoa (H) were also evaluated. All procedures were in an agreement with the requirements for welfare and animal's protection included in Bulgarian legislation.

Semen sexing procedure

The semen sexing was done by application of the TLR7/8 ligand R848 (resiquimod) by adaptation and modification of the protocols of Umehara et al. (2020) and Yotov et al. (2024a) to the fresh ram semen. As it is known (Umehara et al., 2019), the incubation of sperm in a medium with TLR7/8 ligand led to decreased activity of X-bearing sperm and their sinking to the lower layer of the medium. The activity of Y chromosome-bearing sperm is less likely to be decreased, so that they float in the upper layer of the medium.

After removing of the seminal plasma from pools by centrifugation (400g, 5 min, 37°C) the collected spermatozoa were diluted with modified human tubal fluid (mHTF) medium (Umehara et al., 2020) in concentration 200 × 106 spermatozoa per mL. 3 mL of diluted semen from each pool was treated with 0.3 μM R848 (Sigma-Aldrich, Co, St. Louis, MO, USA) and 0.27μM pyruvate (Institute BCN, Spain) followed by incubation at 37ºC for 60 min, and then the upper layer (1 mL) was collected for the Y-bearing spermatozoa. The same procedure was applied to the other 3 mL of diluted semen from each pool, but without pyruvate and followed incubation was 30 min. The X- bearing spermatozoa were collected from the lower layer (1 mL).

Each sample was washed by centrifugation and spermatozoa were suspended with 1 ml freezing extender (Steridyl one step, Minitube, Germany). In accordance with our previous validation of R848 effect on ram semen sexing (Abadjieva et al., 2022), the upper layer contains 74-78% Y-bearing and 22-26% X-bearing spermatozoa whereas the lower layer contains 64-70% X-bearing and 30-36% Y-bearing sperm cells.

Semen cooling, freezing and thawing

Diluted with freezing extender non-sexed and sex-sorted semen was filled in straws of 0.25 ml which were placed of floating freezing rack and were cooled in a refrigerator at temperature 5°C for 5 hours. After cooling, freezing procedure was done through a special freezing unit (Minitube, Germany). The straws stayed upon liquid nitrogen vapours for 15 minutes, then were stored in a liquid N2 container. After freezing, four straws from each part of semen were thawed by placing in a water bath at 37°C for 60 seconds and subjected to CASA analysis.

Statistical analysis

The results were statistically processes by software product Stat.Soft, v.10 (StatSoft Inc., Tulsa, USA). The data are presented as a mean ± standard deviation (SD). After checking for normal distribution of variances by Kolmogorov-Smirnov test, the mean values of the spermatozoa parameters after cooling and freezing were compared with control by the non-parametric Mann-Witney test. Two-ways ANOVA, especially, main effects analyse, was used for the estimation of the effects of factors "sexing" and "extender" and their interaction on the speed parameters of spermatozoa (VCL, VAP and VSL). The differences were considered significant at $P< 0.05$.

Results

The results about the effects of the used diluting media on non-sexing and sexing sperm kinematics are presented in Table 1. The application of freezing extender and main medium for washing of the spermatozoa before semen sexing not affected considerably sperm motility. More significant changes of the motility parameters were observed after the sexing procedure. "Main effects" ANOVA showed that the simultaneously influence of both factors - "extender" and "sexing procedure" significantly affected spermatozoa speed parameters (observed power 0.999; $P=0.0111$, Table 2). The similar trends were observed for all three parameters VCL, VAP and VSL (Figure 1 A, B show these effect on VCL). However, in both studied groups, the strength of the effect was higher for factor extender, especially clear expressed for the sexed spermatozoa group (Table 2).

Table 1. Changes in the motility and kinematic parameters of non-sexed and sexed ram spermatozoa after dilution with different media.

Samples (n=4x3 repetition) with diluting media	Motility (%)				Velocity (%)		Velocity and progressivity (%)			Average values of speed of motile spermatozoa (µm/sec)			Indices (%)			Hyperactive spermatozoa (%)	BCF (Hz)	ALH (µm/sec)
	PR	NP	IM	TM	R	M	S	RP	MP	VCL	VAP	VSL	LIN	STR	WOB			
Non-sexed spermatozoa																		
PhS	99.3 ± 0.65^{A}	0.7 ±0.64^{A}	0.01 ± 0.00^{A}	99.9 ±0.01^{A}	99.2 ±0.76^{A}	0.7 ±0.6^{A}	0.1 ±0.1^{A}	9.2 ±2.5^{A}	90.1 ±3.1^{A}	232.9 ±10.1^{A}	120.2 ±7.8^{A}	59.9 ± 2.4^{A}	26.4 ±1.5^{A}	49.3 ±3.4^{A}	52.2 ±1.9^{A}	82.5 ±4.1^{A}	12.1 ±0.6^{A}	5.2 ±0.01^{A}
Fex	99.9 ±0.0^{A}	0.03 ±0.02^{A}	0.01 ±0.02^{A}	100 ±0.0^{A}	99.9 ±0.03^{A}	0.01 ±0.03^{A}	0.02 ±0.03^{A}	7.5 ±0.3^{A}	92.4 ±0.3^{A}	267.7 ±8.3^{B}	132.5 ±4.4^{B}	64.3 ±2.8^{A}	24.3 ±0.5^{A}	47.4 ±0.7^{A}	49.8 ±0.2^{A}	92.8 ±0.6^{B}	11.2 ±0.01^{A}	6.3 ±0.2^{A}
Sexed spermatozoa																		
mHTF	99.7 ±0.2^{a}	0.3 ±0.^{a}	0.005 ±0.01^{a}	99.9^{a} ±0.01	99.6 ±0.2^{a}	0.3 ±0.1^{a}	0.1 ±0.06^{a}	7.1 ±0.9^{a}	92.5 ±0.7^{a}	261.2 ±10.7^{a}	128.2 ±5.1^{a}	62.4 ±3.1^{a}	24.2 ±0.8^{a}	47.6 ±0.6^{a}	49.5 ±0.3^{a}	90.9 ±1.9^{a}	10.6 ±0.2^{a}	6.2 ±0.3^{a}
mHTF+R848 (X after sexing)	34.5 ±7.2^{b}	52.7 ±7.8^{b}	12.8 ±1.5^{b}	87.2 ±1.5^{b}	29.4 ±8.2^{b}	16.8 ±1.5^{b}	41.0 ±7.7^{b}	5.5 ±1.9^{a}	28.9 ±5.4^{b}	69.9 ±6.4^{b}	41.6 ±1.8^{b}	25.7 ±2.2^{b}	42.8 ±4.1^{b}	61.1 ±2.9^{b}	65.1 ±2.7^{b}	10.8 ±4.2^{b}	6.7 ±0.7^{b}	2.1 ±0.02^{b}
mHTF+R848 (Y after sexing)	42.58 ±3.1^{b}	51.6 ±3.5^{b}	5.8 ±0.7^{c}	94.1 ±0.5^{c}	38.3 ±4.1^{b}	17.4 ±0.3^{b}	38.5 ±2.8^{b}	6.9 ±0.7^{a}	35.6 ±1.2^{b}	80.2 ±3.1^{c}	44.7 ±0.9^{b}	26.4 ±1.9^{b}	38.8 ±2.3^{b}	59.7 ±1.1^{b}	61.3 ±3.4^{b}	12.8 ±2.1^{b}	7.0 ±0.1^{b}	2.2 ±0.03^{b}
Fex + X	96.6 ±4.2^{a}	3.4 ±4.1^{c}	0.04 ±0.03^{ab} ^{c}	99.9 ±0.04^{a}	95.3 ±5.2^{a}	3.5 ±3.6^{c}	1.1 ±1.5^{a}	13.5 ±2.3^{b}	83.1 ±5.8^{c}	183.2 ±13.3^{d}	98.1 ±12.9^{c}	51.5 ±5.1^{c}	29.9 ±2.3^{c}	52.7 ±2.1^{c}	54.9 ±1.2^{d}	58.2 ±7.1^{c}	12.8 ±0.6^{c}	4.4 ±0.1^{c}
Fex + Y	97.4 ±3.6^{a}	2.5 ±3.6^{c}	0.1 ±0.09^{a}	99.9 ±0.09^{a}	96.7 ±4.7^{a}	2.5 ±3.6^{c}	0.8 ±1.1^{a}	11.3 ±3.7^{ba}	86.0 ±7.4^{ac}	206.9 ±11.2^{d}	108.5 ±14.8^{c}	55.6 ±6^{ca}	28.4 ±3.5^{c}	51.0 ±3.2^{c}	53.8 ±2.1^{d}	69.4 ±10.1^{c}	12.0 ±0.8^{c}	4.9 ±0.09^{d}

PhS - physiological solution - 0.9% NaCL; Fex - freezing extender; mHTF- modified human tubal fluid; R848 – resiquimod; PR- progressive motility; NP - non-progressive motility; IM - immotile sperms; R - rapid and M - medium velocity; S - slow, RP - rapid and MP – medium velocity and progressivity; VCL - curve linear velocity; VSL - straight-line velocity; VAP - average path velocity; LIN - linearity; STR - straightness; WOB - oscillation; ALH - amplitude of lateral head displacement ; BCF - beat frequency

$^{A, B}$ - Different letters within the column of non-sexed spermatozoa show the significant difference between the values after dilution with PhS and Fex at P<0.05

$^{a, b, c, d, e,}$ - Different letters within the column of sexed spermatozoa show the significant difference between the values after dilution with mHTF, mHTF+R848 and Fex at P<0.05

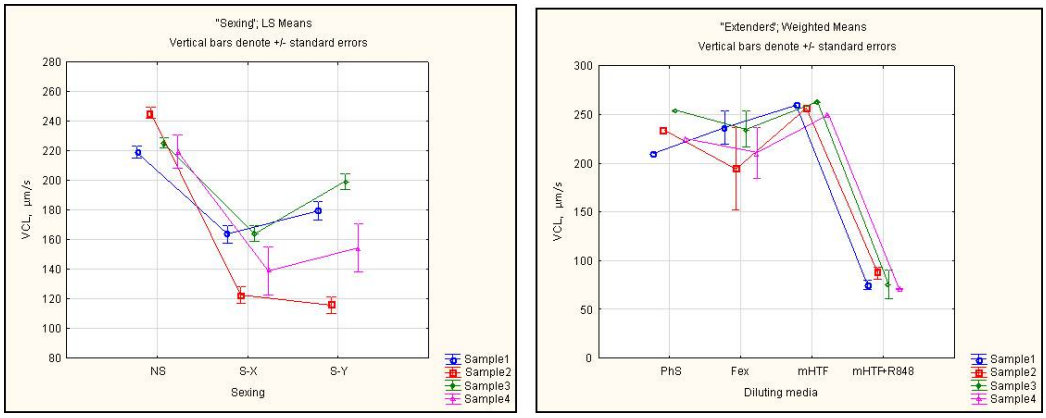

Figure 1. Effects of sexing (A) and diluting media (B) on the VCL of ram spermatozoa
NS - non-sexed spermatozoa, S-X - X bearing sperm fraction; S-Y- Y bearing sperm fraction; PhS - physiological solution 0.9% NaCL; Fex- freezing extender; mHTF- modified human tubal fluid; mHTF+R848 - modified human tubal fluid with R848

Use of Steridyl had the greatest influence, compared to other media (Figure 1B). It affected both non-sexed and sexed spermatozoa, although to varying degrees. In non-sexed spermatozoa, VCL and VAP were significantly higher after dilution with Steridyl, compared to the values obtained in the primary evaluation after dilution with saline (267.7±4.3 vs. 233.1±10.1, P=0.032 and 132.5±4.4 vs. 120.2±7.8, P=0.05, Table 1).

Table 2. Power of extender's effect on the speed parameters of non-sexed and sexed ram spermatozoa (ANOVA main effect analysis)

Effects	Test	Value	P	Observed power (alpha=0.05)
Interaction of Sexing and Extender on the VCL, VAP, VSL for whole group	Wilks	0.000299	0,011011	0.999994
Extender on the VCL for whole group	Wilks	0.001647	0.050166	0.667267
Extender on the VCL for sexed group	Wilks	0.001845	0.027353	0.931995
Extender on the VAP for whole group	Wilks	0.001124	0.042676	0.758951
Extender on the VAP for sexed group	Wilks	0.001194	0.022006	0.976722
Extender on the VSL for whole group	Wilks	0.001735	0.050464	0.654685
Extender on the VSL for sexed group	Wilks	0.002455	0.031554	0.886269

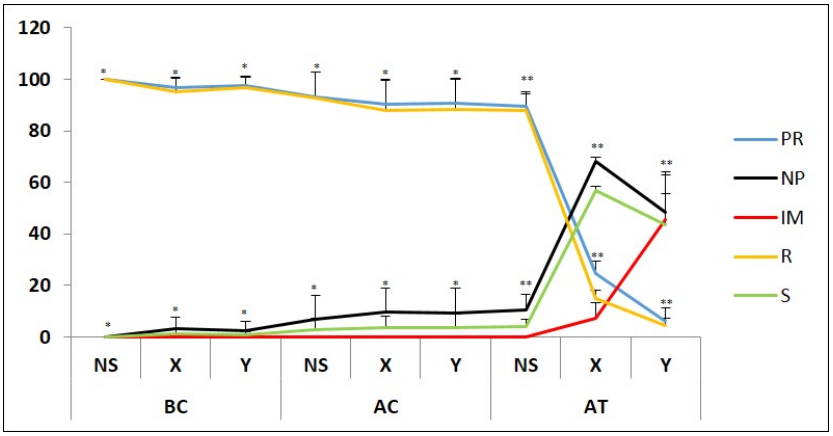

Figure 2. Effect of cooling and freezing on the motility and velocity of non-sexed and sexed ram spermatozoa

NS - non-sexed spermatozoa; X and Y - fractions of sexed spermatozoa; BC - before cooling; AC - after cooling; AT - after thawing; PR - progressive motility; NP - non-progressive motility; IM - immotile spermatozoa; R - rapid velocity; S - slow velocity and progressivity

Values of PR, NP, R and S for the same spermatozoa (NS, X, Y) marked with two asterisks differ at $P<0.05$ according to type of process (BC, AC and AT).

In the sexed spermatozoa, significant changes were observed in the percentages of PR, NP, S and H. Also, in both fractions X- and Y, the speed parameters such as VCL, VAP and VSL increased after dilution with freezing extender. The values of these parameters were close to those recorded for non-sexed spermatozoa (Table 1). During the storage of non-sexed and sexed sperm cells in cooled and frozen condition, the trends for motility changes were similar (Figure 2). Significant decrease in PR and R with increase of NP and S were determined after thawing only, but a percentage of these changes was different. Comparative analysis between both fractions showed significant ($P<0.05$) elevation in the values of PR and R for non-sexed spermatozoa and decrease of NP and S in comparison between X and Y fractions.

The main changes in VCL, VAP и VSL of non-sexed sperms were detected after thawing whereas the indices of movement (LIN, STR and WOB) and ALH started to change with cooling (Figure 3 A, B, C, $P<0.05$). In the X fraction VCL, VAP and VSL were also significantly decreased after thawing, and remaining parameters were changed after cooling (increase of LIN, STR and WOB and decrease of H, ALH и BCF, $P<0.05$) keeping a similar tendency until thawing. In the Y fraction VSL significantly decreased after cooling, while at the same time in VAP, H, ALH и BCF there was only trend to decrease ($P=0.058$ and $P=0.07$). However, after thawing procedure the values of all abovementioned parameters were reduced. There was no change in the movement indices (LIN, STR, WOB)

for cooled or frozen Y fractions. The mean values of speed parameters between non sexed and sexed spermatozoa after thawing differed significantly (P<0.05). At the same time in sex-sorted semen there was an increase of PR, VAP and VSL in X-, compared to Y spermatozoa. Only the value of VCL was significantly (P<0.05) lower in Y than in X fraction.

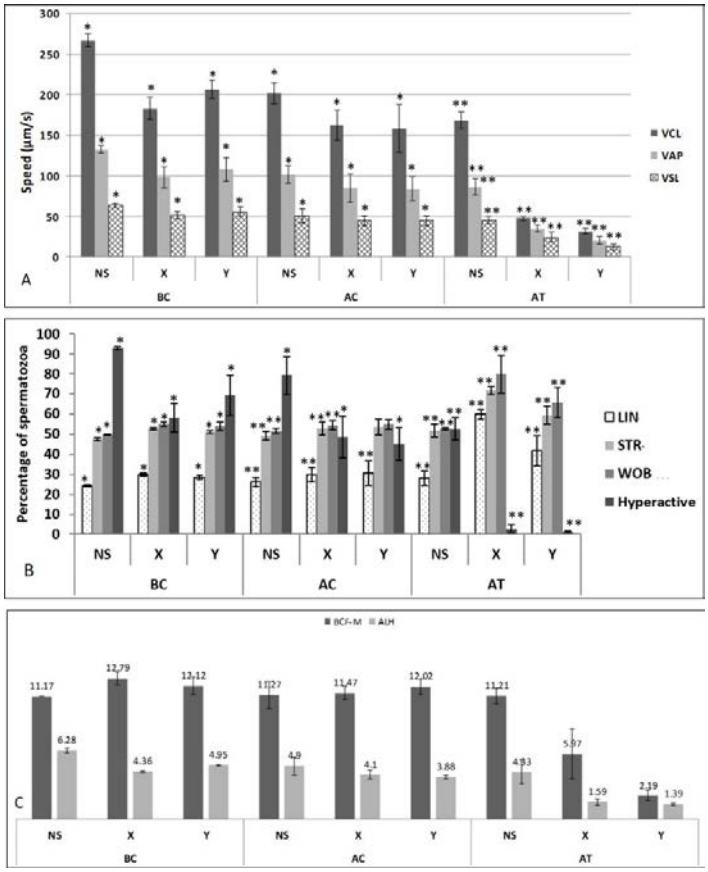

Figure 3. Effect of cooling and freezing on average speed parameters (A), movement indices and hyperactive spermatozoa (B) and amplitude lateral head and beat frequency (C) of non-sexed and sexed ram spermatozoa

NS - non-sexed spermatozoa; X and Y - fractions of sexed spermatozoa; BC - before cooling; AC - after cooling; AT - after thawing; PR - progressive motility; NP - non-progressive motility; VCL - curve linear velocity; VSL - straight-line velocity; VAP - average path velocity; LIN - linearity; STR - straightness; WOB - oscillation; ALH - amplitude lateral head (µm/sec); BCF - beat frequency (Hz) Values for the same spermatozoa (NS, X, Y) marked with two asterisks differ at P<0.05 according to type of process (BC, AC and AT).

Discussion

In the past, assessment of ram sperm quality had been based mainly on subjective evaluation of parameters, such as mass and individual motility, which had proved relationship with semen fertility (David et al., 2015). Computer assisted sperm analysis has allowed an objective and precise evaluation of, not only motility characteristics like progressive and non-progressive motility, rapid and slow spermatozoa, but also the speed parameters, movements indexes, amplitude of lateral head displacement during the movement of motile spermatozoa and number of hyperactive spermatozoa (Valverde et al., 2020). These parameters became important for the ram semen assessment due to numerous last decade investigations, confirming their close relationship with fertility in laboratory (Robayo et al., 2008; Rodriguez- Martinez and Vega, 2013) and in field conditions (Del Olmo et al., 2013; Sinapov and Yotov, 2023).

This study presents for the first time the speed parameters of ram spermatozoa sexed by TLR7/8 ligand R848 and their changes during cooling and freezing of X-and Y-fractions. It was established that interaction of two factors - sexing procedure and use of extenders influences kinematic parameters of ram spermatozoa. In accordance with specifics of R848 sexing protocol, TLR7/8 ligand resiquimod (R848) activates TLR7/8 in X- sperm, resulting in decreased glycolytic activity and ATP production, with a consequent reduction in X sperm motility (Umehara et al., 2019). In confirmation of those are the results of the present study, showing the significant decrease in progressive motility, VCL, VAP, and VSL of X-bearing spermatozoa after sexing. The higher values of these parameters, including TM, for Y-bearing spermatozoa after sexing could be explained by presence of pyruvate in medium for Y-spermatozoa that is a source of energy and an antioxidative agent (Van de Hoek et al., 2022). In contrast, our previous separation of Y-spermatozoa with ligand R848 and creatine showed significant decrease in the total motility for the Y-fraction compared to the whole ejaculate (Yotov et al., 2021). We assume that use of pyruvate is more appropriate than creatine for obtaining the Y-fraction, but future investigations could clarify this question. The suppression of X-spermatozoa motility by TLR7/8 ligand R848 is not stably and can be reversed after removing of R848. In our study the dilution of the sexed spermatozoa with freezing extender "Steridyl" led to significant increase of the motility and speed parameters in both X- and Y-fractions. Moreover, the values became comparable to those of unsexed spermatozoa.

The data about dependence of speed parameters like VCL, VAP, and VSL of ram spermatozoa on the extenders used for dilution has also been reported by Mostafapor and Ardebili (2014). It should be underlined the valuable protective effect of "Steridyl" for unsexed and sexed spermatozoa during the cooling storage. Despite the noticeable trend to decrease the motility and speed parameters in unsexed group, the significant reduction was not observed. However, cooling for 5

hours significantly affected LIN, STR, WOB and number of hyperactive spermatozoa in X-fraction and VSL in Y-fraction, but in the range not more than 10-15%. Important is that cooling did not decrease VAP in either fractions. VAP is the parameter that shows the highest correlation with fertility, and it may be the most useful sperm speed parameter, which can be relied upon for the estimation of sperm fertility (Nagy et al., 2015). The most drastical changes in the kinematic sperm parameters of sexed group appear after freezing. The PR reduced to 24% in X- and 6% in Y-fractions. VCL, VAP, and VSL were reduced by 3-4 times compared to the parameters observed after cooling. The reason for that is a combination of two procedures stressful for sperm - sexing and freezing. The sex-sorting protocol, involving multiple steps and changes in the environment as well as cryopreservation may together lead to motility impairment and oxidative damage of sperm (Vishwanath and Moreno, 2018). The value of PR, speed parameters and number of hyperactive spermatozoa are lower in the post-thawing Y- fraction compared to the X one. These results are in agreement with data reported for the post-thawing Y- fraction separated by BSA column (Yotov et al., 2024b). The mechanism of different cryotolerance of X- and Y-bearing spermatozoa is not clear and has to be investigated in the future.

Conclusions

The present results show that freezing extender "Steridyl one step" can restore the total motility and speed parameters of X- and Y-spermatozoa, reducing after sexing with TLR7/8 ligand R848, close to the primary values and keep them during the cooling at relatively high level comparable with this of unsexed spermatozoa. Regardless protective effect of freezing extender, cryopreservation is very damaging for sexed spermatozoa. In both X- and Y- fractions, the progressive motility, VCL, VAP, VSL and number of hyperactive spermatozoa were adversely affected by freezing. Comparison between fractions allows to conclude that X-spermatozoa are more cryotolerant than Y ones, because they kept higher motility and better kinematic parameters after thawing. Cooling is the better way for short term storage of ram spermatozoa sexed by R848.

Cooled storage can be more successful when sexed fractions are diluted in the appropriate extender that ensures the restoration of the motile and kinematic parameters of spermatozoa after removing of substance R848.

Uticaj hlađenja i smrzavanja na kinematičke parametre spermatozoida ovnova seksiranih modifikovanim protokolom sa TLR7/8 ligandom R848

Stanimir Yotov, Elena Kistanova, Anatoli Atanasov, Boyana Ivanova, Darina Zaimova

Rezime

U okviru ove studije je po prvi put istraživan uticaj sortiranja po polu pomoću TLR7/8 liganda R848, hlađenja i krioprezervacije na pokretljivost i kinematičke karakteristike spermatozoida ovnova. Četiri ejakulata po ovnu (n=2) su sakupljena, spojena i nakon toga podeljena na 8 jednakih delova. Četiri dela su korišćena za određivanje pola, a četiri dela su analizirana kao sperma bez spola. Određivanje pola sperme je urađeno primenom TLR7/8 liganda R848 (resiquimod). Sperma, neseksirana i seksirana po polu, razređena sredstvom za zamrzavanje, hlađena je u frižideru na temperaturi od 5°C tokom 5 sati i nakon toga zamrznuta/odmrznuta. Svaki deo sperme u svakom koraku eksperimenta je podvrgnut kompjuterski potpomognutoj analizi sperme za procenu pokretljivosti i parametara brzine spermatozoida. Nakon seksiranja uočeno je značajno smanjenje kinematičkih parametara u obe frakcije. Razređivanje seksiranih spermatozoida sa sredstvom za zamrzavanje pre hlađenja pouzdano je povećalo progresivnu pokretljivost i parametre brzine kao VCL, VAP i VSL u X- i Y frakcijama i zadržalo ih tokom hlađenja na relativno visokom nivou. Smrzavanje je negativno uticalo na pokretljivost seksiranih spermatozoida. Vrednosti parametara brzine neseksiranih spermatozoida nakon odmrzavanja bile su značajno veće od vrednosti X i Y spermatozoida. Razređivanje odgovarajućim sredstvom za zamrzavanje i hlađenje su najpogodniji pristupi za čuvanje seksiranih spermatozoida ovnova.

Ključne reči: seksiranje sperme ovnova, TLR7/8 ligand R848, kinematika, hlađenje, zamrzavanje

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Conflict of interest
The authors declare that they have no conflict of interest.

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GROWTH RESPONSE, ECONOMIC INDICES, BLOOD PROFILE, AND ORGAN WEIGHT OF PIGS FED REJECTED CASHEW KERNEL MEAL FROM WEANER TO GROWING PHASE

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Abstract: Growth response, economic indices, blood profile, and organ weight of pigs (large white x landrace, n=40, average initial weight = 8.67±0.3kg) fed rejected cashew kernel meal RCKM) were examined from the weaner to growing phase. They were randomly allotted to four groups designated as diet 1, 2, 3, and 4 containing 0%, 5%, 10% and 15% rejected cashew kernel meal respectively in a completely randomized design for eighty-four days. Feed intake differs significantly (P<0.05) across the groups. Pigs offered 5% rejected cashew kernel meal had the highest feed intake with a decreasing trend across those given rejected cashew kernel meal. All economic indices were significant across the groups (P<0.05). Moreover, the heart weight was significantly influenced (P<0.05) by the experimental diet. Pigs fed with 15% RCKM had a significantly higher (P<0.05) heart weight compared with those fed other diets (P<0.05). From the outcome of this investigation, it can therefore be concluded that dietary inclusion of RCKM up to 15% did not trigger any deleterious effects in pigs in terms of growth performance, blood profile, reduced feed cost, increased profit, and economics of gain thus RCKM was well tolerated by the pigs without adverse physiological effects, supported optimal growth, health, and wellbeing of pigs from weaner to grower phase.

Key words: performance, cost, hematology, serum biochemistry, organs

Introduction

In modern animal production systems, the efficient utilization of unconventional feed resources is gaining considerable attention due to the need for sustainable and cost-effective livestock production (Pond et al., 2012). As

traditional feed ingredients face supply constraints and fluctuating prices, the exploration of alternative feedstuffs has become imperative (Alshelmani et al., 2021). Cashew (*Anacardium occidentale L.*) processing generates substantial by-products, among which rejected cashew kernel is noteworthy. Although, the cashew nut itself is widely recognized for its nutritional value, the kernels that do not meet market quality standards, often referred to as rejected cashew kernel meal, have been underutilized (Ojediran et al., 2021).

The utilization of rejected cashew kernel in animal diets, particularly for growing pigs, presents a significant avenue for enhancing feed resource efficiency (Fanimo et al., 2004). Enriched with proteins, energy, dietary fibre, and essential minerals, rejected cashew kernel meal exhibits the capacity to serve as a viable ingredient for a complete or partial substitute for conventional feedstuffs used in livestock diet formulation (Odunsi, 2002; Akande et al., 2015, Ojediran et al., 2022). The incorporation of rejected cashew kernel into livestock diets has the potential to not only mitigate feed expenses but also provide an environmentally sustainable avenue for addressing the management of by-products stemming from the cashew processing sector (Ojediran et al., 2021).

Despite its apparent promise, the use of rejected cashew kernel meal in pig diets requires comprehensive investigation. Assessing its impact on growth performance, and organ development, and other vital organs is crucial to understanding its suitability as a feed resource. Thus, this study addresses the potential of rejected cashew kernel meal as an unconventional feed resource for pig nutrition by evaluating the growth response, economic efficiency, blood profile, and organ weight alterations in pigs to provide scientific insights that can inform effective and efficient strategies for maximizing the utilization of rejected cashew kernel meal, while ensuring animal health and well-being.

Materials and Methods

Location

This research was conducted at the geographical coordinates are approximately 4° 15' East and 8° 07' North. The elevation ranges from 300 to 600 m above sea level. The region experiences an average annual temperature of approximately 27°C and the yearly average rainfall measures around 1,247mm. The study area is characterized by vegetation typical of the derived savannah zone (Ojedapo et al., 2009).

Preparation of test ingredients

The rejected cashew kernel was bought from a local cashew processing firm. Extraneous materials were removed the cashew reject kernel before being milled and added to other feed ingredients for complete ration formulation.

Experimental animal, management and design

Forty 8-week-old weaned pigs (Large white x Landrace) were dewormed, vaccinated and acclimatized for one week prior to the start of the experiment. The pigs were distributed into four groups with ten replicates each in a completely randomized design with each pig as a replicate. The pigs were offered feed and fresh water without restriction throughout the feeding trial. The experiment lasted for 84 days.

Experimental diet

Four diets were constituted to contain 0%, 5%, 10%, and 15% reject cashew kernel meal (RCKM) designated as diet 1, 2, 3, and 4 respectively. The metabolizable energy of the diets ranges from 2732.14 to 2902.44 kcal/kg while the diets were isonitrogenous with 19% crude protein (Table 1).

Table 1. Composition of the experimental diet

Ingredients (%)	DIET 1	DIET 2	DIET 3	DIET 4
Maize	21.00	18.00	12.00	2.00
Soya bean meal	1.00	4.00	7.50	10.00
Groundnut cake	15.00	10.00	5.00	0.00
RCKM	0.00	5.00	10.00	15.00
Corn bran	11.50	11.50	14.00	21.00
#Fixed ingredients	51.50	51.50	51.50	51.50
Total	100.00	100.00	100.00	100.00
Calculated Nutrients				
M. Energy (kcal/kg)	2732.14	2829.21	2891.76	2902.44
Crude Protein	19.58	19.54	19.69	19.72
Ether Extract	5.09	6.17	8.42	10.02
Crude Fibre	8.26	8.64	8.22	8.59

#Fixed ingredients = Palm kernel cake-50.00%, limestone-1.00%, premix-0.25% and salt-0.25%, M. = Metabolizable.

Data collection

Growth performance

Each pig was individually weighed at the commencement of the experiment and subsequently, on weekly basis throughout the experimental duration. The final weight gain of the pigs was also taken at the end of the experiment for the determination of weight gain as the differentials. Moreover, the total feed intake of pigs fed experimental diets was estimated using the difference in feed supply and the leftover feed. The feed conversion ratio was estimated by dividing the total feed intake by the total weight gain (Ojediran et al., 2017).

Economic indices

The economic indices (feed cost, feed cost/kg weight gain (/kgWG), income/kgWG, profit/kgWG and economic efficiency of growth) were calculated by adopting the methods described by Ojediran et al. (2017).

Blood profile

Four (4) pigs of median weight per treatment group were chosen at the end of the study for blood collection. 5ml of blood samples for hematological examination were drawn into labelled EDTA bottles for subsequent analysis of the following haematological parameters: packed cell volume, haemoglobin concentration, erythrocyte count, leukocyte count, mean corpuscular haemoglobin, and mean corpuscular haemoglobin concentration. An additional set of blood samples were also collected into a plain bottle for the following serum biochemical analysis: alanine transaminases (ALT), aspartate amino transaminase (AST) and alanine phosphate (ALP), total protein, albumin, globulin, cholesterol, triglyceride, high-density lipoproteins, low-density lipoproteins, urea, creatinine, and glucose. Blood samples were analysed by adopting the methods described by Ojediran et al. (2021).

Organ Weight

Pigs used for blood collection were slaughtered and analyzed. Prior to slaughtering the pigs, they were weighed and fasted for 12 hours, weighted, and stunned mechanically to subject them to a state of unconsciousness, to facilitate easy slaughtering, and complete bleeding. After slaughtering, a dissection was done on each animal's abdomen to bring out the Gastrointestinal Tract (GIT) and the internal organs. The GIT and the following organs were weighed using a sensitive scale spleen, kidney, liver, pancreas, lungs, heart, whole, and empty stomach. The weights are expressed as a percentage of the fasted weight.

Statistical analysis

Data collected were subjected to a one-way ANOVA using SAS (2003). Significant means were separated by Duncan`s multiple-range test of the same statistical package.

Results

Growth response of pigs fed diets containing rejected cashew kernel meal (RCKM) is shown in Table 2. Feed intake parameters both final and average differ significantly ($P<0.05$) while other parameters such as final weight, total weight change, and feed:gain ratio were not affected ($P>0.05$).

Pigs given diet 2 consumed more ($P<0.05$) feed than those offered other diets with depressed intake as level of RCKM increased in diets 3 and 4.

Table 2. Growth performance of pigs fed rejected cashew kernel meal

Parameters (kg/p)	Diet 1	Diet 2	Diet 3	Diet 4	SEM	P-Value
Initial weight	8.76	8.60	8.68	8.64	0.33	0.99
Final weight	42.84	46.80	46.20	43.06	1.41	0.69
Total weight gain	34.08	38.20	37.52	34.42	1.14	0.49
Average daily weight gain (/d)	0.41	0.46	0.45	0.41	0.01	0.46
Total feed intake	106.59^{c}	121.98^{a}	114.12^{b}	101.59^{d}	1.77	0.00
Average daily feed intake (/d)	1.27^{c}	1.45^{a}	1.36^{b}	1.21^{d}	0.02	0.00
Feed:gain ratio	3.26	3.21	3.05	3.01	0.10	0.81

$^{a\,b\,c}$ Means within rows for different groups with different superscripts differ ($P < 0.05$)
Diet 1: 0% rejected cashew kernel meal; Diet 2: 5% rejected cashew kernel meal; Diet 3: 10% rejected cashew kernel meal; Diet 4: 15% rejected cashew kernel meal; SEM: Standard error of means

Table 3 revealed the economic indices of pigs fed rejected cashew kernel meal (RCKM). All the parameters were affected ($P<0.05$). The feed cost for the control diet was significantly higher than the feed cost for the treatment diets: there was a linear feed cost reduction as the inclusion level of RCKM increased. Consequently, feed cost/kg weight gain followed the same trend as the feed cost. Furthermore, pigs given diet 1 had the highest income/kg weight while the lowest was observed in pigs offered diet containing 5% RCKM (2) while others compared favorably. Furthermore, a similar trend was observed in profit/kg weight gain, and economic efficiency of gain as the lowest values for these parameters were observed in pigs presented the diet 1, while there was an increase in the values of these parameters as the inclusion levels of RCKM increased.

Table 3. Economic indices of pigs fed rejected cashew kernel meal (RCKM)

Parameters	Diet 1	Diet 2	Diet 3	Diet 4	SEM	P-Value
Feed cost (kg)	74.28^{a}	69.19^{b}	64.34^{c}	57.85^{d}	1.55	0.00
Feed cost/kg weight gain	240.80^{a}	221.83^{ab}	196.24^{bc}	173.65^{c}	8.46	0.04
Income/kg weight gain	755.53^{a}	734.49^{b}	738.77^{ab}	749.62^{ab}	3.51	0.01
Profit/kg weight gain	514.52^{b}	512.66^{b}	542.52^{ab}	575.96^{a}	9.02	0.03
EEG	223.37^{b}	232.36^{b}	277.31^{ab}	339.34^{a}	14.24	0.01

$^{a\,b\,c}$ Means within rows for different groups with different superscripts differ ($P < 0.05$)
Diet 1: 0% rejected cashew kernel meal; Diet 2: 5% rejected cashew kernel meal; Diet 3: 10% rejected cashew kernel meal; Diet 4: 15% rejected cashew kernel meal; SEM: Standard error of means; EEG= Economic efficiency of gain

The hematological parameters of pigs fed rejected cashew kernel meal (RCKM) are shown in Table 4. All the parameters were significant ($P<0.05$) across

the treatment groups except for hemoglobin concentration. The white blood cell and lymphocyte count in pigs fed diet containing 10% RCKM (3) was higher than what was observed in diet 1. Similar red blood cell count was noted in pigs given 0% and 5% RCKM, however, the red blood cell count reduced significantly ($P < 0.05$) as levels of RCKM increased. Moreover, significantly ($P < 0.05$) higher PCV of 33.60% was recorded in pigs fed 5% RCKM compared to 28.90% recorded in those offered 0% RCKM, while the lowest PCV values of 25.60% and 21.65% were recorded in pigs fed 10% and 15% RCKM respectively. Mean Corpuscular Volume (MCV) values from pigs fed 5% and 15% RCKM were similar, these values however differ ($P < 0.05$) significantly from the MCV recorded in pigs offered 0% and diet containing 10% RCKM. Furthermore, similar values of 28.75 pg and 28.40 pg were recorded for Mean Corpuscular Haemoglobin (MCH) in pigs fed diets containing 10% and 15% RCKM respectively, these values were higher ($P < 0.05$) than values of 21.00 pg and 23.00 pg which were reported in pigs given 0% and diet containing 5% RCKM respectively. The Mean Corpuscular Haemoglobin Concentration (MCHC) in pigs fed diet containing 10% RCKM was higher ($P < 0.05$) than the similar MCHC values recorded in pigs fed the control diet and diet containing 15% RCKM, while the lowest MCHC value was recorded in pigs fed diet containing 5% RCKM. Values recorded for platelets were significantly ($P < 0.05$) higher in animals offered the RCKM diets compared to the value recorded for pigs fed the control diet.

Table 4. Haematological parameters of pigs fed Rejected cashew kernel meal (RCKM)

Parameters	Diet 1	Diet 2	Diet 3	Diet 4	SEM	P-Value
White blood cell (10^3ul)	10.25^{b}	15.05^{ab}	25.45^{a}	11.95^{b}	2.39	0.04
Red blood cell (10^6ul)	3.02^{a}	3.02^{a}	2.46^{ab}	1.88^{b}	0.18	0.04
Haemoglobin (g/dl)	6.35	6.95	7.05	5.30	0.31	0.18
PCV (%)	28.90^{ab}	33.60^{a}	25.60^{b}	21.65^{b}	1.64	0.03
MCV (fl)	96.45^{b}	111.25^{a}	106.00^{ab}	115.30^{a}	2.57	0.01
MCH (Pg)	21.00^{b}	23.00^{b}	28.75^{a}	28.40^{a}	1.06	0.00
MCHC (Pg)	21.80^{ab}	20.95^{b}	27.35^{a}	24.70^{ab}	0.89	0.01
Platelet (10^4ul)	1267.0^{b}	1577.0^{ab}	2002.0^{a}	1522.0^{ab}	1.78	0.01
Lymphocyte (%)	9.70^{b}	17.70^{ab}	24.75^{a}	11.40^{b}	2.34	0.02

$^{a\,b\,c}$ Means within rows for different groups with different superscripts differ ($P < 0.05$)
Diet 1: 0% rejected cashew kernel meal; Diet 2: 5% rejected cashew kernel meal; Diet 3: 10% rejected cashew kernel meal; Diet 4: 15% rejected cashew kernel meal; PCV = Packed cell volume; MCV = Mean corpuscular volume; MCH = Mean corpuscular haemoglobin; MCHC = Mean corpuscular haemoglobin concentration; SEM: Standard error of means.

The serum biochemical parameters of pigs fed rejected cashew kernel meal (RCKM) are presented in Table 5. Alanine aminotransferase (ALT) was

significantly influenced (P<0.05) by the dietary treatment, pigs fed diet 3 had the highest ALT value of 52.53 IU/L compared to 49.98IU/L that was observed in pigs given diet 1, while significantly lower (P<0.05) ALT values of 46.77IU/L and 41.68IU/L were recorded in pigs fed with diets containing 5% and 15% RCKM respectively. Moreover, the level of Alkaline phosphatase (ALP) was significantly higher (P<0.05) in pigs offered diet 1. A slight reduction in the ALP level was observed in those offered diets 2 and 4, and the lowest ALP level was recorded in pigs fed diets 3.

Significantly higher cholesterol values of 160.87mg/dl and 143.48mg/dl (P<0.05) were recorded in pigs fed diet 3 (10% RCKM) and the control diet (0% RCKM) respectively, while the lowest cholesterol level was observed in pigs offered with diet with 15% RCKM. The highest level of low-density lipoprotein (LDL) was recorded in pigs given diet 3 while the lowest LDL was recorded in pigs offered diet 4. Pigs fed diets 2 and 3 had the highest values (P<0.05) of glucose while the least glucose was in pigs offered diet 4.

Table 5. Serum biochemistry parameters of pigs fed rejected cashew kernel meal (RCKM)

Parameter	Diet 1	Diet 2	Diet 3	Diet 4	SEM	P-Value
ALT (IU/L)	49.98^{ab}	46.77^{b}	52.53^{a}	41.68^{c}	1.34	0.00
AST (IU/L)	117.11	111.05	120.79	118.42	2.95	0.74
ALP (IU/L)	48.77^{a}	45.65^{ab}	33.25^{b}	42.66^{ab}	2.56	0.04
Total Protein (g/dl)	5.39	5..33	6.97	4.78	0.20	0.19
Albumin (g/dl)	2.26	2.52	2.70	2.33	0.74	0.14
Globulin (g/dl)	3.12	2.81	3.28	2.45	0.15	0.21
Cholesterol (mg/dl)	143.48^{a}	123.05^{ab}	160.87^{a}	96.52^{b}	8.91	0.03
Triacylglycerol (mg/dl)	43.73	38.63	43.23	43.03	1.92	0.26
LDL (mg/dl)	97.74^{ab}	84.41^{ab}	117.64^{a}	62.49^{b}	7.87	0.04
HDL (mg/dl)	10.22	5.29	4.38	8.76	1.02	0.12
Urea (mg/dl)	4.61	5.11	5,68	4.71	0.19	0.15
Creatinine (mg/dl)	1.10	1.35	1.27	1.02	0.11	0.73
Glucose (mg/dl)	95.38^{ab}	112.57^{a}	107.36^{a}	84.50^{b}	4.36	0.04

$^{a\,b\,c}$ Means within rows for different groups with different superscripts differ (P < 0.05)
Diet 1: 0% rejected cashew kernel meal; Diet 2: 5% rejected cashew kernel meal; Diet 3: 10% rejected cashew kernel meal; Diet 4: 15% rejected cashew kernel meal; ALT = Alanine aminotransferase; AST = Aspartate aminotransferase; ALP = Alkaline phosphatase; HDL = High-Density Lipoprotein; LDL = Low-Density Lipoprotein; VLDL = Very Low-Density Lipoprotein. SEM: Standard error of means

Table 6 shows the organ weight of pigs offered rejected cashew kernel meal. Heart was significantly affected (P<0.05). Pigs offered diet 4 had the highest weight, while pigs fed the control diet (diet 1), diets 2, and 3 had a statistically

similar weight of the heart, significantly lower (P<0.05) than the value recorded for pigs fed diet 4.

Table 6. Organ weight of grower pigs fed rejected cashew kernel meal

Parameters (%)	Diet 1	Diet 2	Diet 3	Diet 4	SEM	P-value
Spleen	0.14	0.14	0.13	0.14	0.01	0.86
Kidney	0.32	0.37	0.41	0.42	0.02	0.25
Liver	2.33	2.48	2.92	2.75	0.11	0.25
Whole stomach	3.52	2.86	2.85	3.12	0.13	0.22
Empty stomach	0.98	0. 87	0.88	0.82	0.03	0.39
Lungs	0.68	0.72	0.69	0.77	0.02	0.52
Heart	0.39^{b}	0.41^{b}	0.40^{b}	0.55^{a}	0.02	0.01

$^{a\,b\,c}$ Means within rows for different groups with different superscripts differ (P < 0.05)
Diet 1: 0% rejected cashew kernel meal; Diet 2: 5% rejected cashew kernel meal; Diet 3: 10% rejected cashew kernel meal; Diet 4: 15% rejected cashew kernel meal; SEM: Standard error of means

Discussion

This study revealed noteworthy implications for the growth performance of growing pigs when rejected cashew kernel meal was introduced into their diets. Notably, the weight gain (ADG) and feed:gain (FCR), do not vary significantly unlike the feed intake in response to the dietary treatments. This was consistent with the findings of Fanimo et al. (2004) and Oddoye et al. (2011) that reported feeding cashew nut meal to weaner pigs had no significant effect on ADG and FCR. Although, Fanimo et al. (2004) reported a reduction in feed intake in response to the increasing dietary inclusion of cashew nut meal in weaner pigs, while Oddoye et al. (2011) reported similar feed intake in pigs fed cashew kernel meal-based diets and those fed the control diet. It is worthy of note that the elevated feed intake recorded in pigs given diet containing 5% rejected cashew kernel meal (RCKM) may be due to the level of ether extract in RCKM (Ojediran et al., 2021). Consequently, 5% inclusion of RCKM in pig's diet resulted in a moderate level of lipids in the diet which can improve palatability thereby, increasing feed consumption (Kerr et al., 2015). However, high dietary inclusion levels of lipids can result in depressed feed intake (Lin et al., 2013) as seen in pigs fed with diets containing 10 and 15% RCKM respectively.

Economic analysis is a critical factor in evaluating the cost-effectiveness of RCKM inclusion in growing pig diets. From the result of this study, dietary inclusion of RCKM resulted in a concomitant reduction in feed cost. Consequently, this also resulted in reduction in cost per body gain. This findings coincide with that of Nwakpu et al. (1999), Dritz (2012) and Rauw et al. (2020) that the inclusion of alternative feed resources in swine ration significantly reduces feeding cost in

swine production, thereby resulting in huge financial returns. Moreover, the initial reduction in income and profit observed in diet 2, and the subsequent increase in these parameters gave credence to the results of Ojediran et al. (2021) who observed similar trend in income per kilogram weight gain and profit per kilogram weight gain of weaned pigs fed with cashew kernel reject meal. Furthermore, the economic efficiency of gain also increase significantly as the level of RCKM increased in the diet, indicating that dietary inclusion of RCKM in swine ration resulted in improved economic efficiency in line with the finding of Akande et al. (2015) who reported improve production efficiency in laying chickens. The use of alternative feedstuffs with reduced cost and economic gain has been reported (Ojediran et al., 2019).

WBC count obtained from this study ranges between 10.25 * 10^3ul – 25.25 * 10^3ul, while the lymphocyte level was between 9.70% and 24.75%: these values were within the established reference range for healthy pigs (Semiadi et al., 2009; RAR, 2009; Merck, 2023a). This is an indication that the experimental diet did not compromise the immune system of the experimental animals and the animals are immune competent to ward off any disease-causing pathogens they may come in contact with. Moreover, the red blood cell count and heamoglobin concentration recorded in this study falls within the reference range reported for healthy domestic pig by past researchers (Mitruka and Rawnsley, 1977; Etim et al., 2014; Rothwell et al., 2009). This signifies that the experimental diet is rich in essential minerals required in the synthesis of these vital haematological parameters. Furthermore, the PCV, MCV, and MCH were well synthesized in the experimental animal as reflected in the assay of these parameters from this study. This suggests that the experimental animals were not anaemic and that nutrients, oxygen and carbon dioxide were sufficiently circulated throughout the system of the pigs.

Alanine amino transaminase ALT), Aspartate transferase and Alkaline phosphatase (ALP) are liver enzymes that serve as markers used in the determination of liver function. These enzymes are produced by the liver and released into the bloodstream in the event of liver damage or inflammation. (Center, 2007; Derosa and Maffioli, 2017). The level of these liver enzymes recorded in this study were within the reference range for healthy domestic pigs (Okoro et al., 2020; Merck, 2023b). This finding is an indication that feeding rejected cashew kernel meal did not trigger hepatotoxicity in the experimental animals. Moreover, total protein, albumin, and globulin levels which were not influenced by the dietary treatments in the current study imply that RCKM has a rich amino acid profile that supports optimal protein metabolism and tissue regeneration in the experimental animals (Busher, 1990; Meyer and Harvey, 2004). Triacylglycerol level in this study was not affected by including RCKM in swine ration, this is an indication that the energy in the experimental diet is well balanced thereby resulting in effective energy metabolism in growing pigs (Jensen et al,. 1989). Moreover, the level of serum low-density lipoprotein and high-density

lipoprotein from this study is statistically similar across all the treatment groups suggesting optimal lipid metabolism in the test animals, also the test animals are not at risk of cardiovascular disease (Cox et al., 1990).

Organ weight is a valuable parameter that provides insights into the physiological status, growth, development, health, and nutritional efficiency of animals (Li et al., 2021). The relative organs weight obtained from pigs fed with RCKM-based diets and those fed with the control diet are similar and the values were within the range given for normal healthy grower pigs (Amaefule et al., 2020; Elefson et al., 2021). This is an indication that the dietary inclusion of RCKM did not trigger any deleterious physiological response in the experimental animals.

Conclusions

Rejected cashew kernel meal can be included in pigs' diets up to 15% without any deleterious effect on growth performance, blood profile, and organ weight. Also including RCKM in pig's diet up to 15% resulted in the improved economic efficiency of production.

Porast, ekonomski indeksi, profil krvi i težina organa svinja koje su od odbijanja do faze rasta hranjene obrokom sa odbačenim jezgrom indijskog oraha

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Rezime

Ispitivani su porast, ekonomski indeksi, profil krvi i težina organa svinja (jorkšir x landras, n=40, prosečna početna težina = 8,67±0,3 kg) hranjenih brašnom odbačenog zrna indijskog oraha od faze odbijanja do faze rasta. Oni su nasumično raspoređeni u četiri grupe označene kao obrok 1, 2, 3 i 4 koje sadrže 0%, 5%, 10% i 15% odbačenog jezgra indijskog oraha, respektivno, u potpuno randomizovanom dizajnu tokom osamdeset četiri dana. Unos hrane se značajno razlikovao (P<0,05) između grupa. Svinje kojima je ponuđeno 5% brašna odbačenog jezgra indijskog oraha imale su najveći unos hrane sa opadajućim trendom u odnosu na sve grupe koje su dobijale obrok odbačenog jezgra indijskog oraha. Ekonomski indeksi su bili značajni između grupa (P<0,05). Eksperimentalna ishrana je značajno uticala na težinu srca (P<0,05). Svinje hranjene sa 15% RCKM imale su značajno veću (P<0,05) težinu srca u poređenju sa onima hranjenim drugim obrocima (P<0,05). Iz rezultata ovog istraživanja, može se zaključiti da uključivanje RCKM u ishranu do 15% nije izazvalo nikakve štetne efekte kod svinja u smislu performansi rasta,

krvnog profila, smanjene cene hrane, povećanog profita i ekonomičnosti rasta. RCKM su svinje dobro tolerisale bez štetnih fizioloških efekata, podržavao je optimalan rast, zdravlje i dobrobit svinja od odbijanja do faze rasta.

Ključne reči: performanse, cena, hematologija, biohemija seruma, organi

Conflict of interest
The authors declare that they have no conflict of interest.

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FIBRINOUS PERICARDITIS IN SLAUGHTERED PIGS: IMPACT ON WELFARE, GROWTH PERFORMANCE AND CARCASS AND MEAT QUALITY

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Original scientific paper

Abstract: This study aimed to determine the effects of fibrinous pericarditis on blood welfare indicators, performance indices and carcass and meat quality of slaughtered pigs. From a total of 144 examined pig hearts, the presence of fibrinous pericarditis was recorded in 25.69% cases. The presence of fibrinous pericarditis in slaughtered pigs was significantly associated with decreased ($P<0.0001$) blood lactate and glucose levels. The presence of fibrinous pericarditis in slaughtered pigs was significantly associated with reduced average lifetime daily weight gain ($P=0.0042$), live weight ($P=0.0045$), hot carcass weight ($P=0.0045$), cold carcass weight ($P=0.0045$) and lean meat content ($P<0.0001$). Consequently, pigs showing fibrinous pericarditis produced the lower ($P<0.0001$) percentage of „E“ class carcasses, but the higher ($P=0.0079$) percentage of „R“ class carcasses. Pigs without fibrinous pericarditis produced the better meat quality, with the higher ($P=0.0465$) percentage of red, firm and nonexudative meat. In contrast, the presence of fibrinous pericarditis in slaughtered pigs was significantly associated with abnormally elevated final meat pH ($P<0.0001$), high water-holding capacity (lower drip loss; $P<0.0001$) and unfavourable dark colour (higher lightness and yellowness; $P<0.0001$). As a result, pigs showing fibrinous pericarditis produced the highest percentage of dark, firm and dry meat ($P=0.0002$). In conclusion, assessing fibrinous pericarditis at the slaughterline has the potential to serve not only as an indirect indicator of pig health and welfare on the farm of origin but also of growth performance, carcass and pork quality.

Key words: blood metabolites, heart lesions, pig health, pork quality

Introduction

Pig breeders and the meat industry aim to produce animals that have a high percentage of high-quality meat. However, intensive pig production increases the risk of different diseases, including subclinical, which represent one of the most significant problems of animal health and welfare (Čobanović et al., 2019a, 2021, 2024). Since subclinical infections occur without visible signs, on-farm clinical examination cannot be considered an effective method of health and welfare assessment (Dalmau et al., 2014). In addition, when assessing the pig welfare on a farm, the risk of spreading infectious diseases between facilities on a farm and between farms significantly increases. Moreover, the welfare evaluating process on the pig farms is very demanding and takes a long time (Dalmau et al., 2014). On the other hand, the assessment of subclinical lesions in the organs at the slaughterline enables much simpler and more financially profitable data collection to conduct epidemiological research and establish a system for monitoring farm animal diseases (Elbers et al., 1992; Scollo et al., 2017). Examination of the presence and degree of subclinical lesions in clinically healthy pigs at the slaughterline is carried out during a regular postmortem examination of the lungs, pleura, hearts, livers and skin (Elbers et al., 1992; Nielsen et al., 2015; Čobanović et al., 2021).

During pluck examination at slaughterline, the pericardial sac is often damaged and usually incomplete due to professional error of slaughterhouse staff during carcass evisceration (Bottacini et al., 2021). As a result, the presence of exudate cannot be detected, and only visual inspection can reveal lesions in the early chronic stages. Accordingly, the only signs of pericarditis detectable at the slaughterline are fibrin and granulation tissue covering the epicardium (Bottacini et al., 2021). In pigs, fibrinous pericarditis is typically secondary to or connected with primary respiratory infections, with inflammation usually spreading from the lungs due to the lymphohematogenous dissemination of infectious agents such as *Mycoplasma* spp., *Haemophilus* spp., *Actinobacillus pleuropneumoniae*, and *Streptococcus* spp. (Buttenschøn et al., 1997; Leps and Fries, 2009; Čobanović et al., 2019a). It has been found that the coexistence of fibrinous pericarditis and pneumonia and pleurisy in slaughtered pigs, indicating a significant role of pathomorphological changes in the lungs in the pathogenesis of heart lesions (Čobanović et al., 2022). Fibrinous pericarditis negatively affects the pig's health and well-being and causes pain despite the absence of specific clinical signs (Bottacini et al., 2021). Although not usually associated with large economic losses, fibrinous pericarditis can lead to direct losses for the meat industry if pig hearts or whole plucks are discarded at the slaughter line (Ceccarelli et al., 2018). In severe conditions, as is the case of constrictive pericarditis, the reduction of performance may occur in fattening pigs, which can be another underestimated and

understudied economic loss for primary producers and meat industry (Bottacini et al., 2021). Heart failure in fattening pigs can pose a risk of sudden death during stressful procedures on the farm (e.g. vaccination) and during the pre-slaughter period (e.g. loading, transport and unloading) (Bottacini et al., 2021).

Even though the prevalence and impact of subclinical lesions in the lungs and liver on the performance and quality of pig carcasses and meat have been extensively investigated in recent years (Merialdi et al., 2012; Dalmau et al., 2016; Čobanović et al., 2019b, 2021), data on the frequency of fibrinous pericarditis occurrence in slaughtered pigs are scarce. Recent study (Čobanović et al., 2022) in the Republic of Serbia reported the sings of fibrinous pericarditis in 119 out of a total of 1086 slaughtered pigs, with the prevalence of 10.96%. However, there is not enough data in the available scientific literature on the relationship between the occurrence of fibrinous pericarditis and the performance indices, carcass and meat quality of slaughter pigs. To date, only one study (Čobanović et al., 2022) demonstrated a relationship between appearance of heart lesions and reduced performance indices and deteriorations in carcass quality indicators of slaughtered pigs. Therefore, the aim of this research was to determine the effects of fibrinous pericarditis on blood welfare indicators, performance indices and carcass and meat quality of slaughtered pigs.

Materials and Methods

The experiment was conducted in the autumn using 144 fattening pigs referring to four shipments, with approximately 35 pigs per shipment. All pigs were of the same genetic background (Large White × Landrace) sows and Pietrain boars, with an average live weight of 113.50 (±15.32) and about 6 months of age. Animals were sourced from the same family farm, specialised in producing fatteners and operated a single fattening unit with a capacity to finish up to 1000 pigs annually. The fattening unit consisted of 20 pens with fenced outdoor access, each housing around 50 pigs. Pigs were kept on a concrete floor without bedding, at an average stocking density of 0.5 m² per pig. Dry pellets were provided *ad libitum* throughout the fattening period and meals were formulated to meet National Research Council nutrient recommendations for swine (National Research Council, 2012). The farm followed a continuous flow management system, with new pigs introduced monthly and departures to the slaughterhouse occurring every week. There was no parasite control or vaccination program for respiratory diseases in place. All pigs underwent the same pre-slaughter treatment, transport conditions, and lairage on the day of slaughter, following the standard marketing conditions for Southeastern Europe and were slaughtered at the same officially registered and controlled slaughter facility of limited capacity, in line with standard industry practice (Čobanović et al., 2020).

Blood samples were collected from each pig immediately after the onset of bleeding. Blood lactate and glucose concentrations were determined within two minutes using portable devices (for blood glucose: GlucoSure AutoCode, ApexBio, Taiwan; for blood lactate: lactate Scout, EKF Diagnostics, Magdeburg, Germany). Both tubes were labeled with corresponding (slaughter number) to ensure traceability.

The hearts of the slaughtered pigs were removed from plucks at the slaughter line and were meticulously labeled with a ticket containing slaughter number to ensure traceability. Heart and pericardial sack were examined via inspection, palpation and incision for macroscopically noticeable lesions typical for fibrinous pericarditis, using two-point scoring system following the Welfare Quality® protocol (2009): score 0 – no visible signs of fibrinous pericarditis; score 1 – the presence of adhesion between the heart and the pericardium. The percentage of affected hearts was determined by calculating the proportion of pig hearts with adhesion between the heart and the pericardium (Welfare Quality® protocol, 2009). The farm-level score was calculated based on the following thresholds: (i) the warning threshold is triggered if the percentage of slaughtered pigs with pericarditis exceeds 5%; (ii) the alarm threshold is triggered if the percentage of slaughtered pigs with pericarditis exceeds 20% (Welfare Quality® protocol, 2009). A single trained investigator conducted the full assessment of fibrinous pericarditis presence, eliminating potential variations between observers. Gender, slaughter number and identification number from farm were also recorded by the same investigator.

The slaughterhouse personnel provided data on carcass composition, which included hot carcass weight, cold carcass weight, backfat thickness, loin thickness, lean meat content, and carcass quality classes. Live weight at slaughter was estimated based on the determined hot carcass weight, using the equation provided by Vítek et al. (2011): $y = 1.27 * x$, where y represents live weight at slaughter (kg) and x is the hot carcass weight (kg). Estimation of average lifetime daily weight gain was done by subtracting the usual weight of newborn piglet (1.1 kg) from the final weight at the slaughter and then result was divided with average slaughter age (180 days) (Jaeger et al., 2009).

Following evisceration, each carcass included in the experiment was carefully labeled with a carcass ticket to further ensure traceability. The carcasses were then split longitudinally into two equal halves, washed, and weighed 45 minutes postmortem to determine the hot carcass weight. Afterward, the halves were placed in a cold chamber at 4ºC, where they remained for 24 hours. Once the cooling period ended, the carcasses were removed and weighed again to determine the cold carcass weight. The carcass lean meat content (%) was calculated using Two points method approved for Serbia (European Commission, 2008), by measuring loin muscle (*musculus longissimus lumborum*) thickness (shortest distance between the cranial end of the *musculus gluteus medius* and the dorsal

edge of the vertebral canal) and carcass backfat thickness (minimum fat thickness with skin over the *musculus gluteus medius*) in millimeters along the midline of the split carcass using a stainless-steel ruler. Lean meat percentage was then estimated using the following formula as described by Čobanović et al. (2021): y = 65.93356 – 0.17759 * x1 + 0.00579 * x1 – 52.54737 * x1/x2, where y represents the estimated lean meat content (kg), x1 is the backfat thickness (mm), and x2 is the loin muscle thickness (mm). Carcasses were then classified into six classes based on lean meat content, according to the EUROP standard (European Commission, 2008), as follows: "E" (55-60%), "U" (50-55%), "R" (45-50%), "O" (40-45%), and "P" (<40%).

Pork quality measurements were done 45 minutes, 24 hours and 72 hours postmortem on *musculus longissimus lumborum*, at the level of 10^{th} and 11^{th} ribs. The initial and final meat pH (pH_{45min} and pH_{24h}) and temperature (T_{45min} and T_{24h}) measurements were carried out 45 minutes and 24 hours postmortem using a portable pH meter (Testo 205, Testo AG, Lenzkirch, Germany). Subjective pork colour determination was done using National Pork Producer Council (2000) colour standard by three experienced sensorists. Objective pork colour determination was performed using a portable colorimeter (NR110, 3NH Technology CO., Ltd., Shenzhen, China) at six randomly selected points on the surface of the loin muscle and in the core after slicing. The final results were obtained by averaging the *L** (lightness), *a** (redness), and *b** (yellowness) values from six measurements. Water holding capacity was determined via three methods (drip loss, thawing loss and cooking loss) as described by Klauke et al. (2013). Pork quality classes were determined using pH measured 24 hours postmortem, drip loss results and lightness (*L** value) according to Koćwin-Podsiadła et al. (2006): pale, soft, and exudative – PSE meat; red, soft, and exudative – RSE meat; red, firm, and nonexudative – RFN meat; pale, firm, and nonexudative – PFN meat; and dark, firm, and dry – DFD meat.

The statistical analysis was performed using SPSS software, version 23.00 for Windows (SPSS, 2015). Based on the presence of fibrinous pericarditis, the pigs were divided into two groups: (i) pigs without any sign of fibrinous pericarditis (n=107) and (ii) pigs with signs of fibrinous pericarditis (n=37). Student t-test was applied to assess the impact of fibrinous pericarditis on blood welfare indicators, performance indices, and carcass and meat quality parameters. The data were summarised using descriptive statistics, specifically the mean and standard deviation. Fisher's exact test was employed to analyse the distribution of carcass and pork quality classes in relation to presence of fibrinous pericarditis. A P-value of less than 0.05 was considered statistically significant.

Results and Discussion

From a total of 144 examined pig hearts, the presence of fibrinous pericarditis was recorded in 25.69% cases. The frequency of fibrinous pericarditis recorded in the present study was higher than previously established prevalence in Republic of Serbia (10.96%, Čobanović et al., 2022). In different parts of the world, the prevalence of fibrinous pericarditis in slaughtered pigs ranges from: 9% (Bonde et al., 2010) to 13% (Buttenschøn, 1991) in Denmark, 5.66% in Italy (Bottacini et al., 2021), 3.3% (average value) in Portugal, Italy, Finland, Brazil and Spain (Dalmau et al., 2016) and 2.3% in Germany (Mathur et al., 2018). Furthermore, the present investigation revealed that the percentage of fibrinous pericarditis surpassed the 20% alarm threshold established by the Welfare Quality® protocol (2009) for this health indicator. Pathological lesions observed at the slaughterline, such as lung lesions, liver milk spots and fibrinous pericarditis, are commonly associated with substandard production systems (Harley et al., 2012) and suggest significant health and welfare concerns at farm level (Welfare Quality® protocol, 2009).

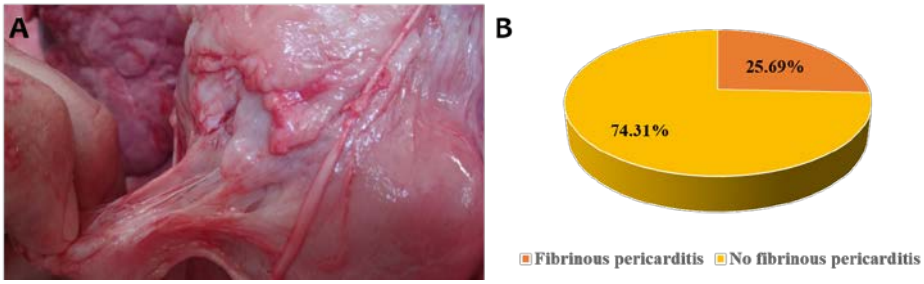

Figure 1. Occurrence of fibrinous pericarditis in slaughtered pigs at the slaughterline

A high occurrence of fibrinous pericarditis in the present study can be ascribed to the fact that pigs raised on family farms with fenced outdoor access are often closed inside the pens during colder months, usually leading to overcrowding, accumulation of dust, hazardous infective agents and noxious gases (ammonia and carbon dioxide) (Done, 1991; Čobanović et al., 2019a, 2019b). High population density can affect pig health by increasing the likelihood of aerosol transmission among pen mates and providing more opportunities for direct nose-to-nose contact with infected individuals (Alawneh et al., 2018; Čobanović et al., 2019a). Furthermore, when pigs are housed in overcrowded, dirty, and isolated barns with improper or irregular manure and sewage disposal, the spread of parasites, viruses, and bacteria is facilitated, contributing to the development of diseases (Čobanović et al., 2019a). Moreover, family farms using continuous flow

management systems face a higher risk of infectious diseases compared to farrow-to-finish farms, as they depend on external sources for weaner restocking, which increases the likelihood of introducing infectious agents from outside the herd (e.g., through carrier pigs) (Stärk, 2000; Done, 1991; Čobanović et al., 2019a).

The effects of fibrinous pericarditis on blood metabolites of slaughtered pigs are shown in Table 1. The presence of fibrinous pericarditis in slaughtered pigs was significantly associated with decreased (P<0.0001) blood lactate and glucose levels. The lower blood glucose and lactate concentrations observed in pigs with fibrinous pericarditis indicate chronic malnutrition, insufficient protein intake, energy deficiency, and dehydration during the period of illness (Šoltésová et al., 2015; Tothova et al., 2016; Čobanović et al., 2019b, 2021). Also, pigs with fibrinous pericarditis, had blood glucose levels below the reference range for the species (Table 1), further suggesting that biochemical changes detected in the present study may be linked to the chronic stages of disease.

Table 1. Mean values (±standard deviation) of blood metabolites in relation to presence of fibrinous pericarditis in slaughtered pigs (n=144)

Item	No fibrinous pericarditis	Fibrinous pericarditis	Reference values (Čobanović et al., 2021, 2024)	P-value
Number of pigs	107	37		
Blood metabolites				
Lactate (mmol/L)	3.89 ± 0.88	1.76 ± 0.53	0.5–5.5 mmol/L	<0.0001
Glucose (mmol/L)	6.85 ± 0.20	3.22 ± 0.32	4.7–8.3 mmol/L	<0.0001

The effects of fibrinous pericarditis on growth performance and carcass quality characteristics of slaughtered pigs are reported in Table 2. The presence of fibrinous pericarditis in slaughtered pigs was significantly associated with reduced average lifetime daily weight gain (P=0.0042), live weight (P=0.0045), hot carcass weight (P=0.0045), cold carcass weight (P=0.0045) and lean meat content (P<0.0001). Consequently, pigs showing fibrinous pericarditis produced the lower (P<0.0001) percentage of „E“ class carcasses, but the higher (P=0.0079) percentage of „R“ class carcasses (Table 2).

Chronic infections that trigger a systemic response and affect the host over an extended period, significantly impact growth rate, feed digestibility, and daily weight gain during the fattening period (Almeida et al., 2020; Ferraz et al., 2020). These effects occur because infections, even in subclinical forms, reduce food intake, hinder nutrient digestion, absorption, and assimilation in the gastrointestinal tract, increase metabolic demands, and promote muscle catabolism while impairing bone and fat tissue synthesis, as well as nutrient transport to target tissues (Čobanović et al., 2019b, 2021).

Table 2. Mean values (±standard deviation) of performance indices and carcass quality characteristics in relation to presence of fibrinous pericarditis in slaughtered pigs (n=144)

Item	No fibrinous pericarditis	Fibrinous pericarditis	P-value
Number of pigs	107	37	
Performance indices			
ADLWG (g)	636.60 ± 7.88	590.50 ± 14.36	0.0042
Live weight at slaughter (kg)	115.60 ± 1.42	107.40 ± 2.58	0.0045
Carcass quality traits			
Hot carcass weight (kg)	91.05 ± 1.12	84.57 ± 2.04	0.0045
Cold carcass weight (kg)	89.68 ± 1.10	83.30 ± 2.00	0.0045
Backfat thickness (mm)	12.64 ± 0.50	17.49 ± 1.08	<0.0001
Loin muscle thickness (mm)	66.92 ± 0.63	67.97 ± 1.46	0.4398
Lean meat content (%)	53.81 ± 0.48	49.46 ± 0.97	<0.0001
Carcass quality classes (%)			
E class	59.81	18.92	<0.0001
U class	18.69	32.43	0.1079
R class	14.02	35.14	0.0079
O class	6.54	8.11	0.7171
P class	0.94	5.40	0.1622

This leads to a reprioritisation and redistribution of nutrients from productive processes like muscle deposition, fat synthesis and bone formation to those with higher nutrient demands, such as plasmatic protein synthesis and the repair of damaged tissues (Čobanović et al., 2019b, 2021). As a result, live and carcass weights, and carcass meatiness decrease (Čobanović et al., 2019b, 2021). Therefore, it can be argued that metabolic disorders associated with fibrinous pericarditis significantly impair pig performance, leading to uneven growth, more days required to reach slaughter weight, reduced final body weight, and a marked decline in carcass quality, ultimately lowering the carcass market value and resulting in considerable financial losses for producers and the pig industry overall (Brewster et al., 2017; Čobanović et al., 2019a, 2021).

The effects of fibrinous pericarditis on pork quality traits of slaughtered pigs are displayed in Table 3. The pork quality analysis showed that pigs without fibrinous pericarditis produced the better meat quality, with the higher (P=0.0465) percentage of RFN meat. This suggests that when animals are free from diseases, dietary energy can be directed toward productive processes, resulting in normal postmortem metabolic functions in skeletal muscles and ensuring the production of high-quality pork (Čobanović et al., 2019b, 2021). Moreover, before-mentioned group of pigs had final meat pH (5.4–5.85 one day postmortem; Honikel, 1999), drip loss (2-5%; Koćwin-Podsiadła et al., 2006) and *L** (lightness) value (42–50; Koćwin-Podsiadła et al., 2006) within the normal range for pork meat, further

suggesting normal postmortem acidification and processes in individuals free from heart lesions.

Table 3. Mean values (±standard deviation) of pork quality traits in relation to presence of fibrinous pericarditis in slaughtered pigs (n=144)

Item	No fibrinous pericarditis	Fibrinous pericarditis	P-value
Number of pigs	107	37	
Physicochemical traits			
pH_{45min}	6.04 ± 0.04	6.17 ± 0.07	0.1381
T_{45min}	38.00 ± 0.14	38.17 ± 0.19	0.5203
pH_{24h}	5.76 ± 0.02	6.00 ± 0.02	<0.0001
T_{24h}	4.29 ± 0.24	4.70 ± 0.44	0.3590
Water-holding capacity traits			
Drip loss (%)	4.65 ± 0.07	2.64 ± 0.66	<0.0001
Thawing loss (%)	6.08 ± 0.28	6.48 ± 0.33	0.4459
Cooking loss (%)	22.26 ± 0.62	23.65 ± 0.66	0.2229
Colour traits			
*L** (lightness) value	48.51 ± 0.30	45.70 ± 0.56	<0.0001
*a** (redness) value	11.68 ±0.65	14.92 ± 1.09	0.0123
*b** (yellowness) value	6.29 ± 0.09	5.28 ± 0.25	<0.0001
Sensory colour	2.89 ± 0.10	3.11 ± 0.10	0.2216
Pork quality classes (%)			
Pale, soft and exudative meat	10.28	0.00	0.0655
Red, soft and nonexudative meat	5.61	5.41	>0.9999
Red, firm and nonexudative meat	70.09	51.35	0.0465
Pale, firm and nonexudative meat	10.28	16.22	0.3780
Dark, firm and dry meat	3.74	27.02	0.0002

In contrast, the presence of fibrinous pericarditis in slaughtered pigs was significantly associated with abnormally elevated final (pH_{24h}) meat pH (P<0.0001), high water-holding capacity (lower drip loss; P<0.0001) and unfavourable dark colour (higher lightness and yellowness; P<0.0001) (Table 3). As a consequence, pigs showing fibrinous pericarditis produced the highest percentage of DFD meat (P=0.0002) (Table 3). Furthermore, aforementioned group of pigs had final meat pH higher than normal range for pork meat (5.4–5.85 one day postmortem; Honikel, 1999), while drip loss and *L** (lightness) value were close to the lower limit of normal range reported for this meat type (Koćwin-Podsiadła et al., 2006).

The findings of this study can be explained by the increased energy demands of severely diseased animals, leading to a depletion of glycogen and adenosine triphosphate (ATP) reserves in skeletal muscles postmortem

(Dailidavičienė et al., 2008; Nenadović et al., 2021; Čobanović et al., 2019b, 2021). This can further explain the lower blood lactate and glucose concentrations observed in pigs with fibrinous pericarditis, resulting in reduced lactic acid in the skeletal muscles and higher meat pH, making it more likely to develop DFD meat (Dailidavičienė et al., 2008; Čobanović et al. 2019b, 2021). In addition to poor processing characteristics, meat from pigs with severe pathological lesions, such as pneumonia, pleurisy, liver milk spots and fibrinous pericarditis, has a shorter shelf life, accelerated autolytic processes, a greater capacity to support bacterial growth, and the highest concentration of total biogenic amines, rendering it unsuitable for storage (Minkus et al., 2004; Dailidavičienė et al., 2009a, 2009b; Čobanović et al., 2017, 2019b, 2021). Based on the results of this study, it can be argued that meat from pigs with fibrinous pericarditis is of lower quality and may not meet the rigorous standards required for placement in the market or production of premium products (Karabasil et al., 2017; Čobanović et al., 2019b, 2021).

Conclusions

The findings of this study revealed a high occurrence of fibrinous pericarditis in slaughtered pigs, signalling a significantly compromised health and welfare on the farm of origin. The presence of fibrinous pericarditis in slaughtered pigs resulted in significant changes in blood metabolite levels, indicating chronic stages of disease. Furthermore, the presence of fibrinous pericarditis in slaughtered pigs significantly reduced growth performance and deteriorated carcass quality characteristics, in terms of lower daily weight gain, live weight, carcass weight and meatiness. Additionally, the presence of fibrinous pericarditis in slaughtered pigs caused a significant deterioration in pork quality traits (abnormally elevated meat pH, increased water-holding capacity, unfavourable dark colour and high percentage of DFD pork). It can, therefore, be concluded that assessing fibrinous pericarditis at the slaughter line has the potential to serve not only as an indirect indicator of pig health and welfare on the farm of origin but also of growth performance, carcass and pork quality. A good feedback system that reports findings of fibrinous pericarditis in pigs from slaughter facilities back to farmers could help in developing, adapting and implementing preventive measures, which in turn would enhance pig well-being standards and improve carcass and pork quality, benefiting both the financial and consumer satisfaction.

Fibrinozni perikarditis kod svinja na liniji klanja: uticaj na dobrobit, indekse performansi i kvalitet trupova i mesa

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Rezime

Cilj ove studije bio je da se ispita uticaj prisustva fibrinoznog perikarditisa na hematološke pokazatelje dobrobiti, indekse performansi i kvalitet trupova i mesa zaklanih svinja. Od ukupno 144 ispitanih svinja, prisustvo fibrinoznog perikarditisa je zabeleženo u 25,69% slučajeva. Zaklane svinje sa fibrinoznim perikarditisom imale su manju (P<0,0001) koncentraciju laktata i glukoze u krvi na iskrvarenju. Prisustvo fibrinoznog perikarditisa kod zaklanih svinja dovelo je do smanjenja dnevnog prirasta (P=0,0042), telesne mase (P=0,0045), mase toplog trupa (P=0,0045), mase hladnog trupa (P=0,0045) i mesnatosti (P<0,0001). Posledično, svinje sa fibrinoznim perikarditisom imale su manji (P<0,0001) procenat „E“ klase kvaliteta trupova, a veći procenat (P=0,0079) „R“ klase kvaliteta trupova. Meso dobijeno od svinja bez fibrinozng perikarditisa imalo je bolji kvalitet i veću (P=0,0465) učestalost crvenog, čvrstog i nevodnjikavog mesa. Nasuport tome, meso dobijeno od svinja sa fibrinoznim perikarditisom imalo je abnormalno visoku pH vrednost (P<0,0001), povećanu sposobnost vezivanja vode (manji kalo ceđenja; P<0,0001) i tamniju boju mesa (veću *L** i *b** vrednost; P<0,0001). Kao posledica, kod pomenute grupe zaklanih svinja utvrđen je veći procenat tamnog, čvrstog i suvog mesa (P=0,0002). Na osnovu rezultata ovog istraživanja, može se zaključiti da ispitivanje prisustva fibrinoznog perikarditisa na liniji klanja svinja može da bude značajan pokazatelj ne samo dobrobiti i zdravstveng stanja svinja na farmi, već i indeksa performansi i kvaliteta trupa i mesa svinja.

Ključne reči: metaboliti krvi, lezije na srcu, zdravlje svinja, kvalitet mesa svinja

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Conflict of interest
The authors declare no conflict of interest.

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PERFORMANCE COMPARISONS ACROSS PIG FARMS

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Abstract: Understanding the dynamics of swine health, including mortality rates during critical stages of development, is essential for improving overall productivity. The aim of this study was an examination of pig farms, focusing on performance metrics such as litter size, mortality rates among different age groups, and weight changes throughout the production cycle. The three farrow-to-finish pig farms from South Bačka District were selected based on their willingness to participate and provide accurate records. The statistical analysis, including one-way ANOVA and post hoc tests, provided further evidence of significant differences among the farms in specific metrics. By analyzing the results in this study, it could be concluded the importance of optimizing breeding, feeding, and health management strategies to enhance swine production efficiency.

Key words: swine, health, performance, productivity, pig farm

Introduction

Swine production plays a crucial role in the global agricultural landscape, providing a significant source of protein for human consumption (Wang and Li, 2024). As the demand for pork continues to rise, optimizing swine health and growth has become increasingly important for producers aiming to enhance efficiency and sustainability (Chatellier, 2021; De Almeida et al., 2024).

Understanding the dynamics of swine health, including mortality rates during critical stages of development, is essential for improving overall productivity. Factors such as litter size, weight at entry and exit points in rearing and fattening phases, and the duration of these phases are pivotal in determining farm efficiency. Previous research has indicated that variations in management practices can significantly influence these performance indicators (Quiniou et al., 2002; Foxcroft et al., 2006; Miller et al., 2012; Craig et al., 2017; Vallet and Miles, 2017; Wijesiriwardana et al., 2022). However, comprehensive comparative

analyses across multiple farms remain limited, highlighting the need for more focused studies in this area.

This paper presents an examination of pig farms, focusing on key performance metrics including litter size, mortality rates among different age groups, and weight changes throughout the production cycle. Furthermore, understanding the implications of these findings may support the development of best practices that enhance swine health, promote growth efficiency, and ensure the sustainable development of the pork industry.

The findings may not only inform producers but also contribute to broader discussions about the future of livestock farming in a rapidly changing world. Ultimately, enhancing swine health and growth is not only vital for economic viability but also for ensuring the resilience and sustainability of agricultural systems as they adapt to new challenges and opportunities (Pfeifer et al., 2022; Vonderohe et al., 2022; Pexas and Kyriazakis, 2023).

Materials and Methods

This study aimed to assess and compare the performance metrics of three farrow-to-finish pig farms: farm A, farm B, and farm C, located in the South Backa District. Capacity of farm A was 300 sows, farm B 1146 sows and farm C 1000 sows.

The research focused on key indicators of swine health and growth, such as litter size, mortality rates, and weight changes throughout the production cycle. Data were collected throughout the year 2023 from each farm, covering multiple breeding cycles. The analysis included the following metrics: the total number of breeding sows and fattening pigs, the average number of piglets per litter, and the percentage of deaths recorded at three stages—suckling, weaning, and finishing. Additionally, the study measured the length of gestation and the lactation period (in days), the average number of litters produced per sow per year, and the weights of pigs at both the entry and exit points of the rearing and fattening phases, all measured in kilograms. The duration of the rearing and fattening phases was also recorded. Data for all three farms were sourced from farm records, including health and growth tracking logs maintained by farm managers.

To analyze the data collected, IBM SPSS Statistics 27 (IBM Corp., Released 2020) was used. A thorough statistical analysis was performed to evaluate the performance metrics across the three farms. This included descriptive statistics to summarize the data, as well as one-way ANOVA to compare the farms and identify any significant differences. A significance level of $p < 0.05$ was set for all statistical tests. If significant differences were found in the ANOVA, Tukey's Honestly Significant Difference (HSD) test was used for post hoc analysis to pinpoint which specific groups differed from one another.

All data were collected in compliance with ethical standards for animal welfare. Farms were selected based on their willingness to participate and provide accurate records. No experimental manipulations were performed on the animals; this study solely utilized existing records to ensure minimal impact on the animals' well-being.

Results and Discussion

The accuracy of data taken from the farms it's verified through consistent monitoring and ground truth data given by farm manager.

The performance metrics of the three farms: A, B, and C are summarized in Tables 1 and 2. The data reveal significant variations in production outcomes, highlighting the influence of management practices on swine health and growth.

Table 1. Key performance metrics across farms

Metric	Farm A	Farm B	Farm C
Number of sows	300	1146	1000
Number of fatteners	1400	31834	28010
Litter size	13	14.38	14.55
Death rate of suckling pigs (%)	10	12.15	12.55
Death rate of weaning pigs (%)	4	3.25	3.4
Death rate of finisher pigs (%)	3	3.54	3.8
Gestation duration (days)	115	115	115
Lactation duration (days)	28	26	26
Farrowing index	2.18	2.34	2.35

Farm B has the highest number of sows (1146) and fatteners (31834), significantly outpacing farms A and C. Farm A, with 300 sows, has the smallest herd size and fattening capacity. Farm B has the largest average litter size (14.38), closely followed by farm C (14.55), while farm A has a lower average (13). The death rates of suckling pigs are highest in farm B (12.15%) and farm C (12.55%) compared to farm A (10%). Farm A has the lowest farrowing index (2.18), indicating fewer litters per sow compared to farms B (2.34) and C (2.35).

Table 2. Weight and duration metrics

Metric	Farm A	Farm B	Farm C
Pig weight entering rearing (kg)	7	6.22	6.9
Pig weight leaving rearing (kg)	28	27.37	29.55
Pig weight entering fattening (kg)	28	27.37	29.55
Pig weight leaving fattening (kg)	105	105.6	109.7
Days in rearing	50	49	49
Days in fattening	105	86	88

The analysis of results in Table 1 and 2, demonstrates significant differences in swine production metrics across the three farms. These variations underline the impact of management strategies on key performance indicators such as litter size, mortality rates, and growth metrics (Quiniou et al., 2002; Foxcroft et al., 2006; Buthelezi et al., 2024).

To provide a comprehensive understanding of the performance metrics across the three farms, a statistical analysis was conducted. This analysis includes descriptive statistics, as well as comparisons using one-way ANOVA to identify significant differences among the farms. Descriptive statistics for key metrics such as litter size, mortality rates, and weight at various stages of production were calculated. The means, standard deviations, and ranges for these metrics are presented in Table 3.

Table 3. Descriptive statistics for key metrics

Metric	Mean	Standard Deviation	Range
Litter size	14.05	0.83	13 - 14.55
Death rate of suckling pigs (%)	11.23	1.24	10 - 12.55
Death rate of weaning pigs (%)	3.88	0.43	3.25 - 4
Death rate of finisher pigs (%)	3.45	0.34	3 - 3.8
Pig weight entering rearing (kg)	6.71	0.46	6.22 - 7
Pig weight leaving rearing (kg)	28.29	0.87	27.37 - 29.55
Pig weight leaving fattening (kg)	106.43	2.07	105 - 109.7

To assess whether the differences among the farms were statistically significant, a one-way ANOVA was conducted for the following metrics: litter size, mortality rates of suckling pigs, weaning pigs, finisher pigs, and weight metrics. The results are summarized in Table 4.

Table 4. One-way ANOVA results

Metric	F-Value	p-Value	Conclusion
Litter size	5.67	<0.01	Significant differences exist
Death rate of suckling pigs (%)	3.89	<0.05	Significant differences exist
Death rate of weaning pigs (%)	0.75	0.50	No significant differences
Death rate of finisher pigs (%)	1.56	0.23	No significant differences
Pig weight entering rearing (kg)	2.45	0.09	No significant differences
Pig weight leaving rearing (kg)	2.17	0.12	No significant differences
Pig weight leaving fattening (kg)	4.12	<0.05	Significant differences exist

A Tukey HSD post hoc test was performed following the ANOVA for metrics that showed significant differences. The results indicated that farm C (14.55) had significantly larger litters than farm A (13) and farm B (14.38), farm A had a significantly lower mortality rate compared to farms B and C and farm C (109.7 kg) had a significantly higher weight compared to farm A (105 kg).

The statistical analysis reveals that while there are significant differences in litter size and suckling pig mortality rates among the farms, not all metrics show significant variability. This indicates that certain management practices have a notable impact on specific performance indicators (Kobek-Kjeldager et al., 2020; Lee et al., 2024).

The analysis of performance metrics across farms A, B, and C reveals significant differences that highlight the critical role of management practices in swine production. Understanding these differences is essential for optimizing health, growth, and overall farm efficiency (Losinge, 2005; Rocadembosch et al., 2016; Alves et al., 2022).

Farm B, with 1146 sows and 31834 fatteners, demonstrates the highest production capacity among the three farms. This substantial scale enables greater economies of scale and resource utilization. In contrast, farm A, with only 300 sows and 1400 fatteners, operates on a much smaller scale, which may limit its overall output and efficiency. The implications of herd size extend beyond mere numbers; larger farms often have access to advanced technologies and specialized management practices that can enhance productivity and animal welfare (Tokach et al., 2016; Maes et al., 2020).

For instance, larger farms may benefit from optimized feeding strategies, better disease management, and more robust health monitoring systems (Dong et al., 2023; Sadeghi et al., 2023). These advantages could explain the improved outcomes observed in farms B and C compared to farm A.

Litter size is a critical factor influencing the efficiency of swine production. Farm B reports the highest average litter size (14.38), followed closely by farm C (14.55), while farm A lags at 13. This difference may reflect variations in breeding

strategies, genetic selection, and overall herd health management (Sadeghi et al., 2023).

Interestingly, the mortality rates of suckling pigs are highest in farms B and C (12.15% and 12.55%, respectively) compared to farm A (10%). This indicates that while larger farms may achieve larger litters, they might also face challenges in managing the health of larger numbers of newborns (Maes et al., 2020; Kobek-Kjeldager et al., 2020). Higher mortality rates can have substantial economic implications, as every lost piglet represents a direct loss of potential revenue (Maes et al., 2020; Dong et al., 2023).

The significant differences in mortality rates underscore the importance of not only maximizing litter size but also implementing effective management practices to ensure the health and survival of piglets (Ward et al., 2020).

The weight metrics observed in the study provide additional insights into the growth performance of pigs across the farms. Farm B shows the lowest entry weight into rearing (6.22 kg), while farm A has a higher entry weight (7 kg). The weight increases during the rearing phase are significant, with farm C achieving the highest weight upon leaving fattening (109.7 kg).

These weight metrics may reflect differences in nutrition and management practices. For example, variations in feed quality, type, and feeding frequency can significantly impact weight gain (Miller et al., 2012; Wijesiriwardana et al., 2022). Farm C's superior performance in weight gain suggests that it may be employing more effective nutritional strategies compared to farms A and B. Understanding the feeding regimens and nutritional management strategies employed by each farm could provide valuable insights for improving weight gain across all farms.

The farrowing index, which indicates the number of litters produced per sow per year, is lowest in farm A (2.18) compared to farms B (2.34) and C (2.35). A lower farrowing index suggests that farm A is not optimizing its breeding program effectively, which may be a consequence of inadequate management practices or reproductive health issues (Rueda López, 2008; Young et al., 2010).

Additionally, the consistency in gestation (115 days) and lactation (28 or 26 days) durations across farms indicates that while these biological parameters are relatively stable, the overall productivity is significantly influenced by management strategies employed post-lactation. Farms that effectively manage the transition from lactation to breeding can potentially improve their farrowing indices (Koketsu et al., 2017).

The statistical analysis, including one-way ANOVA and post hoc tests, provided further evidence of significant differences among the farms in specific metrics. The findings suggest that management practices are a key determinant of performance. Notably, the significant difference in litter size and pig weight leaving fattening illustrates the impact of effective breeding and feeding strategies.

The lack of significant differences in some metrics, such as the mortality rates of weaning and finisher pigs, indicates that while farms may vary in size and

output, certain health management practices may be similarly effective across different operations. This emphasizes the need for a holistic approach to swine health management that can be adapted to various farm sizes.

Conclusions

This study highlights significant variations in swine production metrics across three farms demonstrating the profound impact of management practices on health, growth, and overall productivity. The findings indicate that larger herd sizes, as seen in farms B and C, correlate with improved litter sizes and weight gains, yet also present challenges related to higher mortality rates of suckling pigs. Conversely, farm A, while having a smaller scale, showcased advantages in lower mortality rates but suffered from reduced productivity metrics.

The analysis underscores the importance of optimizing breeding, feeding, and health management strategies to enhance swine production efficiency. Notably, significant differences in litter size and weight metrics emphasize the need for tailored approaches that consider the unique contexts of each farm.

Future research should explore specific management practices that contribute to the observed differences, with the aim of developing best practices that can be implemented across various farm sizes. By leveraging insights from this comparative study, the swine industry can work towards enhancing productivity, improving animal welfare, and meeting the increasing demand for pork in a sustainable manner.

Poređenje performansi na farmama svinja

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Rezime

Razumevanje dinamike zdravlja svinja, uključujući stopu mortaliteta tokom kritičnih faza razvoja je od suštinskog značaja za poboljšanje ukupne produktivnosti na farmi svinja. Cilj ovog istraživanja je bio ispitivanje performansi na farmama svinja, fokusirajući se na metrička poređenja kao što su veličina legla, stopa mortaliteta među različitim starosnim grupama i razlike u težini prasadi tokom proizvodnog ciklusa. Tri farme svinja iz Južnobačkog okruga izabrane su na osnovu njihove spremnosti da učestvuju i pruže tačne evidencije o produktivnosti na njihovim farmama. Statistička analiza, uključujući jednosmernu ANOVA-u i post hoc testove, pružila je dodatne dokaze o značajnim razlikama među farmama

u performansama. Analizom dobijenih rezultata može se zaključiti važnost optimizacije strategija uzgoja, hranjenja i upravljanja zdravljem kako bi se poboljšala efikasnost proizvodnje svinja.

Ključne reči: svinja, performance, produktivnost, farma svinja

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Conflict of interest
The authors declare that they have no conflict of interest.

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DETERMINATION OF SOME QUALITY PARAMETERS OF HONEY BEE FEED

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Original scientific paper

Abstract: Due to an increasing deficiency in feed for bees, beekeepers increasingly tend to use commercial industrial sugar (sucrose) in the nutrition of bee colonies. In the bees nutrition, sugar can be used as liquid sugar - syrup (diluted in water in different ratios). In addition, sugar can be used to make sugar dough (candy paste). Beekeepers often add enzymes or acids to invert the sugars and speed up the process with additional heating. When the bees are fed syrup that has not been overheated no serious problems arise. But in a case when overheated or multiple heated syrups and the syrup hydrolyzed/inverted by inorganic acids are used in the nutrition the occurrence of hydroxymethylfurfural (5-hydroxymethyl-2-furancarboxaldehyde-HMF) which can shorten a life span or cause bee mortality. The purpose of this study is to determine concentrations of HMF, reducing sugars, sucrose, free acids and pH value of solid and liquid feed for bees in the samples of candy paste and syrup, with a particular focus on HMF. During the 2012-2021 period in the laboratory of the Veterinary Specialized Institute "Kraljevo" (VSI "Kraljevo"), in Kraljevo 42 samples of candy paste, 23 samples of enzyme hydrolyzed sugar (syrup) and 6 samples of acid and/or temperature inverted or diluted sugar (syrup) were analyzed. Out of 68 food samples analyzed for the presence of HMF 8 samples (11.76%) were positive for the presence of HMF in concentrations higher than 40 mg/kg.

Key words: feed for bees, hydroxymethylfurfural, sugars, free acidity, pH

Introduction

Owing to increasing climate changes, intensive farm management, single-crop farming, deforestation, and large-scale melioration a considerable part of an area of bee pasture is being reduced (Quinlan et al., 2023). Taking into consideration a lack of food sources, beekeepers more and more turn to various alternatives wishing to replace nectar and pollen by artificial food. When you type "honey bees feeding" into your browser you receive lots of articles and research papers whose number has, over the last 20 years, increased from about 100 to over 700 annually (Graph 1). Therefore the growing interest both of beekeepers from the region and some firms to produce suitable food for bees that will in certain periods of the year replace nectar in bee nutrition is not surprising.

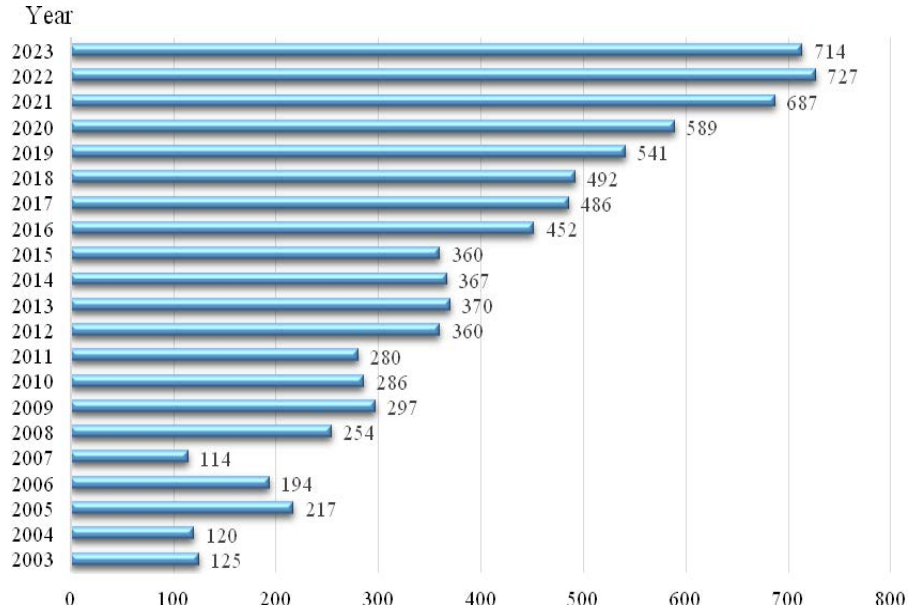

Figure 1. Number of published articles in period of last 20 years (this data was taken from the Science Direct database using one research query "Honey bees feeding")

Qualitatively and quantitively balanced nutrition plays a primary role in maintaining healthy and strong bee colonies. To satisfy their needs the bees collect nectar, honeydew, pollen, resinous substances and water. Nectar provides them with carbohydrates to satisfy their energy needs and pollen provides them with proteins for growth and development. Other nutritive components obtained mostly from nectar and pollen (lipids, minerals, vitamins, enzymes, colored and odorous

substances, etc.) supplement their nutrition (Standifer et al., 1977). However, in certain months, years and/or in some regions the food for bees can be scarce or even absolutely lacking. Hence, beekeepers have to provide additional sources of carbohydrates to feed their bee colonies (Haydak, 1970; Krainer et al., 2016; Paray et al., 2021; Tsuruda et al., 2021). In bee nutrition sugar is the most often used (obtained from sugar beet or sugar cane) in the form of liquid food. It is diluted with water in different ratios and used in the form of liquid food. Besides this, high fructose corn syrup or starch syrup are used as well (Brodschneider et al., 2010; Krainer et al., 2016). A solid carbohydrate food – sugar dough (candy paste) can be made out of sugars. Jevtić et al. (2003) reported that for the development of colonies in spring, the addition of one candy paste in 3 different terms in the preceding winter period had the most favorable effect. The addition of two candy pastes in three terms harmed spring development and resulted in the death of colonies.

The beekeepers often add enzymes or acids for sugar hydrolysis/inversion and accelerate a whole process by extra heating. The additional carbohydrated food increases the strength of colony, prevents starvation and decreases excessive winter losses (Emsen and Dodologlu, 2014). In our climate conditions additional nutrition is necessary in late summer and in early autumn when bees make their winter supplies but also in the spring when the colonies are stimulated to produce a large amount of brood. Beekeepers often produce syrup themselves by mixing sugar and water and by adding some acidifying agents such as acetic and lactic acids (Standifer et al., 1977).

If the bees are fed a syrup that has not been overheated and that has been supplemented with enzymes (*invertase*) for hydrolysis there are no big problems with bee products or with bee health. The additional feeding with sugar syrup or solid food, particularly with that of a weak quality, can lead to several problems. The first is the adulteration of honey and the second is that harmful substances found in syrup or candy pastes (5-hydroxymethyl-2-furancarboxaldehyde/hydroxymethylfurfural/HMF, acids) can get into the honey (Kanelis et al., 2022; Schrenk et al., 2022). Adulteration of honey can occur by feeding the bees with sucrose syrup which is made from sugar beet or by adding industrial sugar syrups obtained from starch: by a thermal, acid or enzyme inversion (Ruiz-Matute et al., 2007). An excessive use of these feeds in the time of bee major pasture can get into a final product honey and is considered as adulterated honey (Bogdanov, 2006; Guler et al., 2007; Guler et al., 2014; Dodd et al., 2024). If in bee nutrition we use an overheated or multi-heated sugar syrup or the one hydrolysed by acids (most often inorganic) in which there occurred a formation of HMF, a life span of a worker bee can be shortened while higher consumption may even be lethal (Schrenk et al., 2022). HMF is a cycling aldehyde that is most often created by dehydration of fructose and glucose in an acid environment. At first, the presence of HMF was an indicator of adulteration of

honey but nowadays it is primarily the indicator of heating (decrystallisation) and freshness of honey. The concentration of HMF in honey increases by shelf life and besides temperature and time of storing it depends also on the type of honey, its pH, share of acids and humidity. A high quantity of HMF (more than 100 mg/100g honey) in the first 15 days of feeding does not negatively affect the worker bees. However, further feeding by such food (15-30 days) causes an abrupt dying of the cells of midgut and increases the mortality of such fed bees (Gregorc et al., 2020). After 30 days of feeding only a control treatment had more than 20% alive bees while other treatments (100-1500mg/100kg) had below 10% survived individuals. Somewhat higher mortality of bees fed by syrup containing 150 mg/kg HMF was reported by LeBlanc et al. (2009), who said that 50% adult individuals died after 19 days of feeding by such syrup. Ceksteryte and Racys (2006) think that bees can metabolize a low quantities of HMF in stored food. Of the similar opinion are also Jachimowicz and El Sherbiny (1975) who think that concentration of 30-48 mg/kg HMF ought not be harmful for worker bees.

Numerous studies have shown that HMF can be toxic for adult bees (Schrenk et al., 2022). Bailey (1966) determined that honey which has been stored for a long time contains an increased quantity of HMF, causing increased bee mortality in contrast to fresh honey. Jachimowicz and El Sherbiny (1975) determined that concentration of HMF of 30 ppm did not cause a significant mortality in adult individuals. In addition, they determined that a high quantity of HMF (150 ppm) in syrup which was produced by acid hydrolysis significantly increases bee mortality in bees fed by this syrup. Taking this into consideration, Krainer et al. (2016) studied the impact of HMF on mortality of larvae, pupa and juvenile bees of 2-22 days old. They confirmed that concentration of HMF of 5-750 ppm does not affect increased mortality but that concentration of 7500 ppm leads to complete dying of larvae and pupae.

An even bigger problem with HMF can arise during preparation of a solid feed. During the preparation of syrup for candy pastes it is often overheated or inverted by inadequate substances (inorganic acids), therefore in this way a great quantity of HMF can be generated in feed.

Smodiš Škerl and Gregorc (2014) fed 50 bees in cages with candy pastes from commercial production. The concentration of HMF in candy pastes was less than 10 mg/kg HMF in the first two candy pastes, 437 in the third and in the fourth 914.6 mg/kg HMF. Bees fed by candy pastes with lower concentration of HMF lived significantly ($p < 0.0001$) longer (up to 27 days) compared with the bees fed candy pastes with higher HMF (20-24 days).

Materials and Methods

In the period from 2012-2021 in the laboratory of the Veterinary Specialised Institute "Kraljevo" (VSI "Kraljevo"), in Kraljevo, the samples of candy paste by enzyme hydrolysed (inverted) sugar and acids and/or by temperature inverted or diluted sugar were analysed in order to study 5 parameters of quality of food for bees: HMF, reducing sugars, sucrose, free acids and pH (Table 1). The parameters were analysed in 42 samples of sugar dough, 23 samples of enzyme hydrolysed sugar (syrup) and 6 samples of acidifying and/or by temperature inverted or diluted sugar (syrup) in laboratory conditions. The samples were delivered by beekeepers and firms from 13 municipalities of central and southwest Serbia. Since neither in the Republic of Serbia nor in the world, there is a legal framework which regulates the way of production, parameters of quality and declaration for bee feed (Paray et al., 2021; Tsuruda et al., 2021), each sample was analysed for one or more different parameters of quality at the personal request of each customer.

Table 1. Type of feed for bees, number of laboratory analysis done per types of food and number of processed parameters of quality

Food	HMF	Reducing sugar	Sucrose	Free acids	pH
Candy paste	42	17	16	5	1
Enzyme inverted sugar (syrup)	20	23	22	3	12
By acids and/or by temperature inverted or diluted sugar (syrup)	6	6	6	-	3
Σ	68	46	44	8	16

The quantitative method for the determination of HMF, reducing sugars (%) and sucrose content (%) were analyzed following the International Honey Commission (Bogdanov et al., 2002).

The free acidity was determined by the titrimetric method, using NaOH and HCl. Ten grams of each sample was dissolved in 75 ml distilled water in 250 ml beaker. It was stirred with the magnetic stirrer, the pH electrodes immersed in the solution and pH recorded. It was titrated with 0.1M NaOH to pH 8.30 (a steady reading should be obtained within 120 sec of starting the titration; in other words, complete the titration within 2 minutes). Recorded the read to the nearest 0.2 ml when used a 10 ml burette and to 0.01 ml if the automatic titrator has sufficient precision. Results were expressed as mEq/kg.

The values of pH were read on the pH meter Scholar 425 (Corning, USA).

Results and Discussion

Neither in the Republic of Serbia nor in the world there is a legislation on the quality of feed for bees (Paray et al., 2021; Tsuruda et al., 2021). There are some legal frameworks regarding the quality and health safety of honey and some bee products. If it is known that honey is the best food for honey bee then the other food as well should meet the standards set for honey. It must be admitted that all the parameters of honey quality cannot be applied to the food for bees as well because some feeds (sugar dough) are produced mainly from sucrose. Thus the application of the Rule book on Quality of Honey and Other Bee Products (2015) can refer only to some of the parameters: HMF, reducing sugars and free acids.

Out of total of 68 samples analysed in the laboratory for the presence of HMF the highest level of HMF was determined in the samples of candy paste for bees (Table 2).

Table 2. HMF concentration in feed for bees (mg/kg)

	Number of samples	Mean ± SD	Number of samples >40 mg/kg	Min	Max	Coefficient of variation (%)
Candy paste	42	31.18±52.62	6	0.3	212.16	168.76
Enzyme inverted sugar (syrup)	20	17.40±33.23	2	0.3	127.49	190.99
By acids and/or by temperature inverted or diluted sugar (syrup)	6	2.6±0.87	0	1.67	3.5	33.96
Σ	68		8			

The highest average concentration of HMF was determined in candy paste and the largest number of samples (6) contained more than 40 mg/kg HMF. By a laboratory analysis of enzymatically inverted sugar yielded slightly better results, as it had 55.6% less HMF compared to candy paste. Only two out of twenty laboratory processed samples had more than 40 mg/kg HMF. In the samples of acidifying and/or by temperature inverted or diluted sugar an average concentration of HMF was 2.6 mg/kg, while none of the samples had a concentration higher than 40 mg/kg. It should be taken into consideration that the smallest number of samples of this feed was analysed but it is obvious that the feed was prepared in a suitable way meaning there was neither overheating nor repeated heating.

If sucrose is used for inverting the syrup and if there is no overheating and/or repeated heating HMF does not exceed the allowed limits. In all the samples

of syrup obtained at different temperature values (35-65 ºC), the concentration of HMF was lower than 4.32 mg/kg (Radovanović et al., 2017).

A concentration of reducing sugars indicates the level of hydrolysis of sucrose in table sugar rendered into glucose and fructose. This parameter was determined in a total of 46 food bee samples (Table 3).

Table 3. Reducing sugars in feed for bees (%)

	Number of samples	**Mean±SD**	**Number of samples <60%**	**Min**	**Max**	**Coefficient of variation (%)**
Candy paste	17	17.88±9.34	17	7.76	40.0	52.24
Enzyme inverted sugar (syrup)	23	54.10±11.28	15	29.12	69.54	20.84
By acids and/or by temperature inverted or diluted sugar (syrup)	6	50.57±19.05	3	13.17	63.32	37.67
Σ	46		35			

All analysed samples of candy paste (17) had an increased content of sucrose, what was expected. A candy paste is made by mixing a ground table sugar and inverted syrup so that sucrose is prevalent in it but there was also a candy paste that contained up to 40% reducing sugars. An average value of the quantity of reducing sugars in syrup obtained by enzyme hydrolysis of sugar shows that in the majority of syrups prepared in this way, the value is close to the Rulebook on Quality of Honey and Other Bee Products (2015). According to this rulebook, a flower honey should not have less than 60 g/100g honey while forest honey should not contain less than 45g/100g. The same values were obtained by acid and/or by temperature inverted or diluted sugar where only one sample had a reduced content of reducing sugars and the others were close to or above the values defined in the Rulebook on Quality of Honey and Other Bee Products (2015). In the research conducted by Frizzera et al. (2020) it was determined that bees fed syrup made from water and sucrose lived longer than bees fed a mixture of water, glucose and fructose (1:1:1).

The honey bee hydrolyses sucrose to reducing sugars by enzyme invertase for which process they spends energy and proteins (Frizzer et al., 2020). The wish of beekeepers to help the bee in this activity seems to be justifiable but not always successful and sometimes even unneccessary (Frizzer et al., 2020). The concentration of sucrose is highest in candy paste while it is considerably lower in enzyme and acid and/or temperature inverted or diluted sugar (Table 4).

Table 4. Sucrose in feed for bees (%)

	Number of samples	Mean±SD	Number of samples >10%	Min	Max	Coefficient of variation (%)
Candy paste	16	73.54±6.5	16	65.06	85.99	8.84
Enzyme inverted sugar (syrup)	22	10.44±9.16	7	2.35	38.4	87.74
By acids and/or by temperature inverted or diluted sugar (syrup)	6	11.85±13.43	1	3.18	38.98	113.3
Σ	44		24			

In the samples of enzyme invert sugars the values of sucrose were increased in several samples, in seven samples the value was higher than 10% while in other samples the values were close to those prescribed by the Rulebook on Quality of Honey and Other Bee Products (2015). Only in one sample of acidified and/or by temperature inverted or diluted sugar the value of sucrose was higher than 10%.

Kanelis et al. (2022) searching for methods to determine adulterated honey proved that feeding the bees by different kinds of food can lead to honey free of sucrose when the bees are fed invert syrup. Somewhat more sucrose was found in the honey produced by bees fed candy paste (3.2%). The highest value of sucrose was determined in the honey made by bees fed sugar syrup (6.2%).

The presence of free acids was determined in 8 samples of food for bees (Table 5).

Table 5. Free acids in feed for bees (mEq/kg)

	Number of samples	Mean±SD	Number of samples >50% mEq/kg	Min	Max	Coefficient of variation (%)
Candy paste	5	11.34±12.96	-	1.6	34.1	114.28
Enzyme inverted sugar (syrup)	3	11.38±15.7	-	1.7	29.5	137.92
By acids and/or by temperature inverted or diluted sugar (syrup)	-					
Σ	8		-			

The values of the abovementioned parameter in the samples of candy paste and invert syrup were below the maximum values permitted by the Rulebook on Quality of Honey and Other Bee Products (2015). The increased acidity in food for bees can arise either by acidification or fermentation of food (alcoholic and vinegar fermentation). During this period, in the samples analysed in a laboratory, regarding the production of food for bees no such issue occurred so that the results were satisfying as well. During the production of food for bees, particularly during inversion, a special attention should be paid to the pH value. An acidification of food for bees by citric or hydrochloric (HCl) acid to pH 2.8 significantly decreases surviving of worker bees in relation to bees fed by acid-free syrup (Frizzera et al., 2020).

When they consume food honey bees reduce its pH value to about 4. This also pertains to royal jelly whose pH value at the moment of secreting is about 7 (Muresan and Buttstedt, 2019). Before it is given as food to larvae royal jelly is mixed with fatty acids produced in maternal glands by which process the pH is reduced to 4-4.5. A majority of the total number (16) of samples analysed on this parameter had the appropriate values (Table 6).

Table 6. pH in feed for bees

	Number of samples	**Mean±SD**	**Min**	**Max**	**Coefficient of variation (%)**
Candy paste	1	4.73			
Enzyme inverted sugar (syrup)	12	4.79±1.29	3.07	6.7	26.95
By acids and/or by temperature inverted or diluted sugar (syrup)	3	4.41±0.87	3.41	4.92	19.71
Σ	16				

By inverting acidic food (pH 2) a large quantity of HMF is being generated, hence, almost only 10 minutes after boiling point the concentration of HMF reaches 1786.7 mg/l. After 40 minutes of boiling the level of HMF increases to 14366.7 mg/l what is very harmful for bees. At higher values (pH 3 and 4) such high quantities of HMF (Frizzera et al., 2020) were not observed.

Conclusions

Upon the laboratory analysis of samples of feed for bees in the laboratory of the Veterinary Specialised Institute “Kraljevo” there are several conclusions to be drawn:

The greatest interest both of beekeepers and firms engaged in the production of feed for bees was shown for the value of HMF. It is obvious by the number of requests for this kind of analysis, 68 samples/analyses in total. Somewhat less interest was shown for reducing sugars and sucrose, while the least interest was shown for the presence of free acids and the pH value.

In eight samples of feed for bees (6 samples of candy paste and 2 samples of acidifying and/or by temperature inverted or diluted sugar) determined value of HMF was higher than 40 mg/kg HMF what can cause problems in bees feeding (weakening and/or dying of bees).

The quantity of reducing sugars in feed produced for bees is significantly lower than those prescribed for honey. Out of 46 samples analysed in 8 samples of enzyme invert sugar and in 3 samples of acidifying and/or temperature inverted or diluted sugar, their value was higher than 60%.

High CV values show that in practice there are no standard methods of food preparation that are used in feeding bees.

The concentration of free acids and pH value of feed for bees were within the values permitted by Rulebook on Quality of Honey and Other Bee Products (2015).

Utvrđivanje određenih parametara kvaliteta u hrani za pčele

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Rezime

Zbog sve većeg nedostatka hrane za pčele, pčelari sve više pribegavaju upotrebi konzumnog šećera (saharoze) u ishrani pčelinjih zajednica. U ishrani pčela šećer se može koristiti kao tečna hrana, sirup (rastvara se vodom u različitim odnosima). Takođe se od šećera može praviti šećerno testo (pogača). Želeći da ubrza proces razgradnje saharoze, pčelari često u vodeni rastvor šećera dodaju sredstva za invertovanje (enzime ili kiseline), a ceo proces ubrzavaju dodatnim zagrevanjem. Ukoliko se pčele hrane sirupom koji nije pregrevan i kome su za invertovanje dodati enzimi (*invertase*) ne javljaju se veći problemi. Ukoliko se u prihrani koristi pregrevan ili više puta zagrevan sirup i onaj koji je hidrolizovan/invertovan kiselinama (najčešće neorganskim) može doći do stvaranja hydroxymethylfurfural-a (5-hydroxymethyl-2-furancarboxaldehyde-HMF) koji skraćuje životni vek pčela

radilica, a pri višim koncentracijama dovodi do njihovog uginuća. Cilj rada bio je utvrđivanje fizičko-hemijskih parametra, čvrste i tečne hrane za pčele u uzorcima šećernog testa i sirupa, prvenstveno HMF-a. U periodu od 2012-2021. godine u laboratoriji Veterinarskog Specijalističkog Instituta "Kraljevo" (VSI "Kraljevo"), u Kraljevu laboratorijski je ispitivan sadržaj HMF, redukujućih šećera, saharoze, slobodnih kiselina i pH hrane za pčele. Ispitano je 42 uzorka šećernog testa, 23 uzoraka enzimski hidrolizovanog šećera (sirupa) i 6 uzoraka kiselinama i/ili temperaturom invertovanog ili samo rastvorenog šećera (sirupa). Od ukupno 68 uzorka hrane za pčele analiziranih na prisustvo HMF-a, u 8 uzoraka (11,76%) utvrđeno je prisustvo HMF-a u koncentraciji višoj od 40 mg/kg.

Ključne reči: hrana za pčele, hodroksi metil furfural, šećeri, slobodna kiselost, pH

Author Contributions
K.M., N.N., J.Ć. and G.J. conceived of the study, and participated in its design and coordination and helped to draft the manuscript. V.K., R.M. and M.D. carried out the lab work. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest
The authors declare no conflict of interest.

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NUTRITIONAL QUALITY OF DONKEY MILK DURING THE LACTATION

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Abstract: The Domestic Balkan and Banat donkey (*Equus asinus asinus*) are native donkey breeds primarily raised in the Special Nature Reserve 'Zasavica', Serbia. The study's objective was to analyze the composition of donkey's milk during the lactation period (12th to 30th week of lactation), regardless of the donkey's breed. The investigated donkey milk composition is characterized by a low content of dry matter, protein and fat and a high amount of lactose, compared to the milk of other dairy animals. The study revealed that content of dry matter ranged from 7.20 to 9.52%, fat ranged from 0.10 to 1.00%, protein ranged from 1.17 to 2.07%, lactose ranged from 4.52 to 6.71%, ash ranged from 0.28 to 0.50% and pH ranged from 6.82 to 7.46. Dry matter, fat, and ash content progressively lowered during lactation, with some oscillations. Milk protein and lactose content were not affected during the lactation stage. The pH value increased progressively during lactation with slight oscillations during the 18th and 21st week of lactation. Statistically significant differences between lactation weeks were established only for lactose content and pH values.

Key words: donkey milk, milk composition, lactation

Introduction

Donkey milk is considered to have the potential for use as a dietetic food and substitute for human milk. Sensory properties, a high amount of lactose and a low amount of fat and proteins make donkey milk closely similar to mare and human milk (Garhwal et al., 2023; Živkov Baloš et al., 2023; Albertos et al., 2022; Prasad, 2020; Marchis et al., 2018; El-Hatmi et al., 2015). Compared to ruminant milk, donkey milk has a lower content of dry matter, ash, fats and proteins,

especially caseins, and a significantly higher content of lactose, and whey proteins (Martini et al., 2018, 2021; Salari et al., 2019). On the other hand, compared to human milk, donkey milk has a lower content of dry matter and fat, a similar content of lactose, and proteins and a higher content of ash (Martini et al., 2021; Swar, 2011). The data from the literature suggested significant differences in the chemical composition of donkey milk concerning the contents of individual components.

The composition of milk can be attributed to the breed or type, diet, age, lactation number, season of the year, farming system, climate and health status of the animal (Bhardwaj et al., 2020; Nayak et al., 2017; Kučević et al., 2016). The donkey breed and seasonal variations are factors that have the greatest influence on milk composition (Kaskous and Pfaffl, 2022; Bhardwaj et al., 2020). In addition, diet is a very important factor that can affect the content of individual components in milk (Miraglia et al., 2020; Burden and Bell, 2019; Raspa et al., 2019; Lazarević et al., 2017). The donkeys are considered a seasonal polyestrous species, therefore, when adequate planning in the breeding seasons milk could be available throughout the year (Giosuè et al., 2008). The lactation period of donkeys ranges from 180 to 350 days (Giosuè et al., 2008; Guo et al., 2007; Salimei et al., 2004). Lactation peak usually occurs between 4 and 5 weeks from parturition (Centoducati et al., 2012).

The contents of individual components of donkey milk have been determined worldwide, and data from the literature suggested certain differences between donkey breeds. These include Halari donkey from India (Garhwal et al., 2023), Cyprus (Aspri et al., 2019) and Arcadian from Greece and Cyprus (Massouras et al., 2020), Amiata from Italy (Sarti et al., 2019; Salari et al., 2019; Ragona et al., 2016), Jiangye and other Chinese breeds (Li et al., 2018; Guo et al., 2007), Ragusana from Italy (Malacarne et al., 2019; Giosuè et al., 2008; Salimei et al., 2004), Martina-Franca from Italy (Salimei et al., 2004), Zamorano-Leonese from Spain (Albetros et al., 2022), Tunisian breeds (Aroua et al., 2018; El-Hatmi et al., 2015), Domestic Balkan from Serbia (Lazarević et al., 2017) and Litoral Dinaric from Croatia (Ivanković et al., 2009).

A significant number of donkeys worldwide are not systematized but are classified as domestic donkeys. The situation is similar in Serbia, where the domestic Balkan donkey breed is officially recognized. However, genetic research indicates that this breed has different genetic profiles in Serbia. Thus, besides the domestic Balkan donkey, it is believed that there is another breed in Serbia, namely the Banat donkey (Urošević, 2022). In the area of the "Zasavica" special nature reserve, both genotypes are breeding, and from this reserve, the idea and project of separating and forming the Banat donkey breed originated (Mandić et al., 2022). Donkeys have been used as working animals in Serbia for centuries, primarily in agriculture and transport. There is not enough data on the composition of the milk

of domestic breeds, and current Serbian regulations on milk quality (Rule Book, 2017) do not cover donkey milk.

This research focused on the composition and nutritional analyses of Balkan domestic and Banat donkeys, during the middle stage of lactation to obtain data important for donkey milk standardization.

Material and Methods

Milk samples

Milk samples (18 composite samples) were collected from healthy Balkan and Banat jennies from The Special Nature Reserve "Zasavica", Serbia. The donkeys were reared outdoors in extensive livestock farming. The animals grazed on natural pastures and drank water from natural springs. After parturition, and during the first two months, all the milk was left for the foal. Starting from 45-60 days after parturition, the jennies move from pasture to pen, together, that is, there are no individual meals. Their diet includes alfalfa hay and corn. Alfalfa hay is the basic coarse feed, in addition, they feed on raw or spent meadow grass or alfalfa. They get corn in the morning and the afternoon. Raw milk samples were obtained by hand milking of 15 jennies, aged 3 to 15 years. Samples were taken every 3 weeks, from May to October 2023 (12th to 30th week of lactation). The foal stayed with mothers and they were separated only a few hours before milking. The composite milk samples (3 composite samples), of an average volume of 100 ml were collected in glass bottles. The samples were transported under refrigerated conditions (4º C), on the same day to the laboratory of Scientific Veterinary Institute „Novi Sad“, Novi Sad.

Physicochemical analysis

Nutritional analysis of milk was performed in terms of dry matter, fat, total proteins, ash, and lactose content. Dry matter was determined by the oven method (ISO 6731) at 102° C, and ash content by the muffle furnace method at 550^0 C (AOAC 930.30). Fat content was determined by the Gerber method (ISO 11870, 2009). Proteins were determined by the Dumas method (ISO 14891), and total acidity (pH) was measured by a pH meter. Lactose was determined by an HPLC (Jakšić et al., 2022).

Statistical analysis

All measurements were made in triplicate, and the results were expressed as mean value ± standard deviation. To analyze variations of the results, ANOVA was used: Single factor including F-test. The analysis was performed using the software package Microsoft Office Excel 2007. Differences between the means with probability $p<0.05$ were accepted as statistically significant.

Results and Discussion

The composition of Balkan and Banat donkey milk samples are summarized in Table 1. The gross composition is characterized by a low content of dry matter (DM), protein and fat and a high amount of lactose compared to the milk of other dairy animals. The obtained data were compared with the results reported by other authors from our and other countries. Our study revealed that in all examined donkey milk samples, the percentage of dry matter ranged from 7.20 to 9.52% (mean value 8.49%). These results are consistent with other literature data, according to which DM content is in the range from 7.50 to 10.40% (Salimei et al., 2004; Guo et al., 2007; Ivanković et al., 2009; El-Hatmi et al., 2015; Ragona et al., 2016; Lazarević et al., 2017; Li et al., 2018; Aroua et al., 2018; Aspri et al., 2019; Sarti et al., 2019; Salari et al., 2019; Malacarne et al., 2019; Massouras et al., 2020; Albetros et al., 2022).

Table 1. Descriptive statistics of physicochemical properties of Balkan and Banat donkey milk

Composition	Range	Mean values	SD	RSD (%)
DM (%)	7.20 – 9.52	8.49	0.54	14.56
Fat (%)	0.10 – 1.00	0.41	0.28	67.99
NFDM (%)	6.20 – 8.70	8.08	0.63	7.85
Protein (%)	1.17 – 2.07	1.69	0.25	14.56
Lactose (%)	4.52 – 6.71	5.38	0.66	12.36
Ash (%)	0.28 – 0.50	0.37	0.06	16.32
pH	6.82 – 7.46	7.19	0.17	2.43

SD – standard deviation; RSD – relative standard deviation; DM – dry matter; NFDM – non-fat dry matter

Fat content (mean value 0.41%) of all analyzed donkey milk samples ranged from 0.10 to 1.00% and was and consistent with other literature data according to which fat content is in the range from 0.16 to 1.30% (Salimei et al., 2004; Guo et al., 2007; Giosuè et al., 2008; Ivanković et al., 2009; El-Hatmi et al., 2015; Ragona et al., 2016; Lazarević et al., 2017; Li et al., 2018; Aroua et al., 2018; Aspri et al., 2019; Sarti et al., 2019; Salari et al., 2019; Malacarne et al., 2019; Massouras et al., 2020; Albetros et al., 2022). Milk fat in cow milk is considered a risk factor for human health because of its high content of saturated fatty acids. Donkey milk is characterized by low fat content and low energy value, as well as the content of fatty acids that are desired in a balanced human diet (Živkov Baloš et al., 2023). The content of fat in donkey milk is influenced by breed, nutrition, milking technique, milking intervals, lactation stage and number of lactations (Guo et al., 2007).

The range of protein content (mean value 1.69%) in the investigated donkey milk samples were between 1.17 and 2.07%, similar to the results of other authors (from 1.22 to 2.14%) (Salimei et al., 2004; Guo et al., 2007; Giosuè et al., 2008; Ivanković et al., 2009; El-Hatmi et al., 2015; Ragona et al., 2016; Lazarević et al., 2017; Li et al., 2018; Aroua et al., 2018; Aspri et al., 2019; Sarti et al., 2019; Salari et al., 2019; Malacarne et al., 2019; Massouras et al., 2020; Albetros et al., 2022). Casein fraction in the total protein content of donkey milk is considerably lower as compared to bovine milk, which contributes to the low allergenicity of donkey's milk (Kaskous and Pfaffl, 2022; Bhardwaj et al., 2020; Martini et al., 2021). The content of whey proteins, lactose and casein in donkey milk is similar to that in human milk, though significantly different from cow, goat and camel milk. The casein/whey protein ratio is higher in donkey milk than in human milk. On the other hand, casein/whey protein ratio in ruminant milk is four times higher than that of donkey milk, and seven times higher as compared with human milk (Vincenzetti et al., 2017; Derdak et al., 2020). Gubić et al. (2016) reported that Balkan donkey milk represents a source of antibacterial proteins such as lysozyme and highly digestible proteins such as whey protein, α-lactalbumin and lactoferrin.

According to research data from Serbia and other countries, the lactose content in donkey milk ranges from 5.90 to 7.23±0.243% (Salimei et al., 2004; Guo et al., 2007; Giosuè et al., 2008; Ivanković et al., 2009; Lazarević et al., 2017; Ragona et al., 2016; Aroua et al., 2018; Sarti et al., 2019; Salari et al., 2019; Malacarne et al., 2019; Massouras et al., 2020; Albetros et al., 2022). Our study revealed that in all examined donkey milk samples, the percentage of lactose ranged from 4.52 to 6.71% (mean value 5.38%). The lactose concentration in donkey milk is similar to that in human milk. However, compared to bovine milk, donkey milk contains significantly more lactose (Martini et al., 2018; Ragona et al., 2016). Lactose stimulates intestinal absorption of calcium and phosphorus contributing to the homeostasis of these elements, which is especially important for bone mineralization and prevention of osteoporosis (Martini et al., 2021; Vincenzetti et al., 2017; Nayak et al., 2017; Massouras et al., 2017). Lactose is a nutritional factor and a potential probiotic because it stimulates the development of intestinal enteroflora (Yvon et al., 2016).

The mean ash content in investigated donkey milk samples was 0.38%, with a range of 0.28 to 0.50% (Table 1), similar to the results of other authors (from 0.32 to 0.51%) (Salimei et al., 2004; Guo et al., 2007; El-Hatmi et al., 2015; Ragona et al., 2016; Lazarević et al., 2017; Li et al., 2018; Aroua et al., 2018; Sarti et al., 2019; Salari et al., 2019; Malacarne et al., 2019; Massouras et al., 2020; Albetros et al., 2022). Cow's milk has two times higher ash content than donkey's milk. The lower mineral concentration in donkey milk has a favorable effect on the function of the kidneys since they are not burdened by minerals (Martini et al., 2018). Calcium, phosphorus, sodium, and magnesium contents are higher compared to human milk, but lower than those in cow, buffalo, goat and sheep

milk (Bhardwaj et al., 2020). Calcium, manganese, iron, selenium and zinc contents are similar in horse and donkey milk, whereas the concentrations of sodium, potassium and copper are significantly different (Bilandžić et al., 2014). Compared to bovine milk, calcium/phosphorus ratio in donkey milk is more suitable for human nutrition (Malacarne et al., 2019; Li et al., 2018; Massouras et al., 2017). The most prevalent microelement in donkey milk is zinc, with concentrations above the levels detected in human milk. Donkey milk contains twofold amount of copper as compared with human and cow milk. The content of selenium in donkey milk is ten times higher than that in cow and sheep milk, and seven times higher than in human milk (Albertos et al., 2022).

Average pH value of milk samples was 7.19 and ranged from 6.82 to 7.46, similar to the results of other authors (7.01- 7.35) (Guo et al., 2007; El-Hatmi et al., 2015; Aroua et al., 2018; Aspri et al., 2019; Salari et al., 2019; Malacarne et al., 2019; Albetros et al., 2022). Aroua et al. (2018) reported that donkey milk has an almost neutral pH (7.09), whereas the pH values of cow and goat milk are 6.65 and 6.50, respectively. The almost neutral pH of donkey milk is attributed to the lower casein and phosphate levels (Salimei et al., 2004).

Table 2. Physicochemical properties of Balkan and Banat donkey milk during the 12th to 30th week of lactation (mean values ± standard deviation)

Week of lactation	DM (%)	Fat (%)	NFDM (%)	Protein (%)	Lactose (%)	Ash (%)	pH
12	9.03±0.70	0.70±0.28	8.33±0.42	1.56±0.11	5.20±0.49	0.39±0.04	7.12±0.17
15	8.55±0.01	0.65±0.00	7.90±0.01	1.80±0.04	5.28±0.30	0.39±0.01	7.15±0.08
18	8.18±0.91	0.50±0.44	7.68±1.31	1.62±0.23	4.60±1.52	0.43±0.07	6.94±0.11
21	8.63±0.32	0.37±0.21	8.26±0.49	2.01±0.07	5.49±0.80	0.37±0.05	7.24±0.03
24	8.70±0.08	0.17±0.06	8.53±0.14	1.51±0.37	6.26±0.40	0.34±0.07	7.24±0.03
27	8.06±0.46	0.23±0.06	7.83±0.41	1.63±0.06	4.69±0.14	0.30±0.03	7.43±0.03

DM – dry matter; NFDM – non-fat dry matter

Physicochemical properties of Balkan and Banat donkey milk during the 12th to 30th week of lactation are displayed in Table 2. and Figure 1. An analysis of variance indicates that the values between the groups, defined based on the lactation period of the donkeys, did not show statistically significant differences for the examined parameters: protein, dry matter, non-fat dry matter and ash, since obtained values for the F-test were lower than the F-critical ones. Statistically significant differences in the measured lactose and pH values were noted between the formed groups. The F-test values were significantly higher than the F-critical ones. This is in correlation with the results in Table 2., where the measured values

of lactose and pH values at different weeks of lactation have a slightly wider range of measured values.

Dry matter, and consequently and non-fat dry matter, showed a progressive lowering, with some oscillations, from the 12. week of lactation (9.03%) to the 27. week of lactation (8.06%) (Table 2). Ivanković et al. (2009) reported that the lactation stage of Littoral-Dinaric donkeys significantly influences DM content in the milk. The highest dry matter concentrations were established at the early lactation stage. A significant positive correlation was observed between the milk yield, dry matter and fat contents. Malacarne et al. (2019) reported a progressive decrease in DM content throughout the lactation period, with minor oscillations from the early to the late lactation period. However, Martini et al. (2014) concluded that DM in the milk of Amiata donkeys did not change significantly during lactation.

A progressive lowering trend was found for fat (from 0.70 to 0.23%) (Table 2., Figure 1.). The fat content varies throughout the donkey's lactation period, whereby it shows a decreasing tendency, especially after the 150th lactation day (Massouras et al., 2017; Martemucci and D'Alessandro, 2012). However, Lazarević et al. (2017) state that fat content in Balcan donkey milk did not vary significantly within the period of lactation and was slightly higher in the early lactation. Salimei et al. (2004), Guo et al. (2007) and Aspri et al. (2019) reported a similar trend in fat content decrease in donkeys' milk after 100 days of lactation.

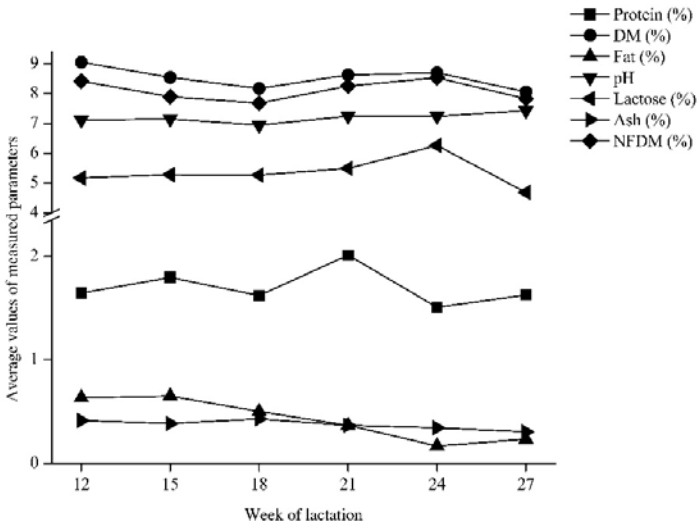

Figure 1. Lactation stage effect on physicochemical properties of Balkan and Banat donkey milk

The protein content did not vary significantly during the middle and late lactation (Table 2., Figure 1.). The research of Martini et al. (2014) conducted on Amiata donkeys revealed a progressive decrease in milk protein content during first 6 months of lactation, and consequent stabilization of the concentration at 1.50%. Similar results are reported by Lazaravić et al. (2017). They state that the highest concentrations of protein were determined during the early lactation period, and gradually decreased from the beginning to the end of the lactation. Gubić et al. (2016) in their study analyzed proteins of Domestic Balkan donkey milk during the lactation period (from the 45th to the 280th day). They reported that protein reached the highest value of 1.92% on the 60th day of the lactation stage. The protein concentration decreased until the end of the lactation period and reached 1.40%. Barbosa Dos Santos et al. (2023) concluded that donkey age and lactation stage affect milk composition. Milk total solids and protein fraction content decrease with the lactation stage. Aspri et al. (2019) reported a decreasing trend in the protein level of donkey milk during lactation. They concluded that this phenomenon may occur due to the different expression of genes responsible for milk protein synthesis during the lactation period.

The lactose content was influenced by the stage of lactation (Table 2., Figure 1.) and was uniform in the 12th and 15th weeks, but there was a decrease in the 18th week. After that, in the 21st and 24th weeks of lactation, it increased slightly, but in the 27th week, the content significantly decreased. Martini et al. (2014) reported that lactose levels in the milk of Amiata donkeys decreased during the first 60 days of lactation and then remained constant until the end of lactation. Malacarne et al. (2019) in their research conducted on the Ragusano donkey breed established a progressive decrease of lactose content in donkey milk throughout the lactation period.

The ash content showed a progressive lowering (from 0.39 to 0.30%) (Table 2., Figure 1.). Malacarne et al. (2019), concluded that ash content in the milk of Ragusano donkeys (Italy) decreases during lactation. These results are comparable with the results of Albertos et al. (2022) reported for Zamorano-Leonese donkeys (Spain). Martini et al. (2014) reported a decrease in ash content in the milk of Amiata donkeys during the lactation period. Salimei et al. (2004) based on the results of their study about milk quality from Martina Franca and Ragusana donkey breed concluded that ash content was constant throughout the experimental period (28 - 150 days after parturition). According to the results of Lazaravić et al. (2017), statistically higher ash content was observed in the middle period of lactation, compared to other stages of lactation.

A progressive increasing trend was found for pH value (from 7.12 to 7.43) (Table 2., Figure 1.). Salari et al. (2019) in their study about milk from the Amiata donkey breed, noticed that the pH increased in the late lactation stage. They stated that an increase of pH value at later lactation stages is associated with a decrease in casein level, and an increase of urea content in the milk (Salari et al., 2019).

However, Aspri et al. (2019) stated that the pH value of donkey milk does not significantly change during the lactation period.

Conclusions

Banat and Balkan donkey milk did not show a large variability of physicochemical properties. The homogeneity of the milk produced during lactation is important when donkey milk is intended for sensitive categories of consumers. Donkey milk is not standardized, so it is necessary to develop regulations that would regulate its quality. Further research is needed on donkey's nutrition and milking. In addition, thanks to favorable climatic conditions and soil characteristics, Serbia has great potential for developing extensive livestock production, including donkey farming. The breeding of donkeys and the production of milk, due to the modest needs of donkeys in nutrition and care, can be additional and profitable activities for farmers.

Nutritivna vrednost mleka magaraca tokom laktacije

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Rezime

Domaći balkanski i banatski magarac (*Equus asinus asinus*) su autohtone rase magaraca, koje se prvenstveno gaje u Specijalnom rezervatu prirode „Zasavica“ u Srbiji. Cilj studije je bio da se analizira sastav mleka magarica obe rase, u periodu laktacije (od 12. do 30. nedelje laktacije). Sastav ispitivanog magarećeg mleka karakteriše nizak sadržaj suve materije, proteina i masti i visok sadržaj laktoze, u poređenju sa mlekom ostalih životinja namenjenih za proizvodnju mleka. Ispitivanjem je utvrđeno da je sadržaj suve materije varirao od 7,20 do 9,52%, masti od 0,10 do 1,00%, proteina od 1,17 do 2,07%, laktoze od 4,52 do 6,71%, pepela od 0,28 do 0,50% i pH vrednosti od 6,82 do 7,46. Sadržaj suve materije, masti i pepela se progresivno smanjivao tokom laktacije, uz izvesne oscilacije. Sadržaj proteina i laktoze nije bio pod uticajem faze laktacije. pH vrednost se progresivno povećavala tokom laktacije sa blagim oscilacijama tokom 18. i 21. nedelje laktacije. Statistički značajne razlike posmatrano po nedeljama laktacije, utvrđene su samo za sadržaj laktoze i pH vrednosti.

Ključne reči: magareće mleko, sastav mleka, laktacija

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Conflict of interest
The authors declare that they have no conflict of interest.

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TOTAL PHOSPHORUS CONTENT IN MEAT PRODUCTS ON THE MARKET OF THE REPUBLIC OF SERBIA AND REGULATORY COMPLIANCE

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Abstract: This study focused on monitoring the phosphate levels in meat products on the Serbian market over a two-year period and evaluating the producers' compliance with regulations on additive usage. During the mentioned period, 74 different meat products (222 samples in total) were analyzed, including finely and coarsely minced cooked sausages. The analysis was conducted using the standard method (SRPS ISO 13730:1999) at the Institute of Animal Husbandry, Belgrade – Zemun laboratory. Content of total phosphorus expressed as P_2O_5 varied between 2.01 g/kg and 6.98 g/kg for finely minced sausages, while for coarsely minced sausages, it ranged from 4.13 g/kg to 7.97 g/kg. The results show that producers incorporate phosphates in line with the specified limits.

Key words: additives, total phosphorus, phosphates, meat products, Serbia

Introduction

Different foods contain a wide range of nutrients, some of which are naturally present, while others are added for specific functional properties. In the food industry, where it is essential to produce goods that meet consumer expectations regarding appearance, texture, aroma, and taste while ensuring a high level of food safety (Prica et al., 2012; Abedi-Firoozjah et al., 2024), the inclusion of substances used to modify certain technological, sensorial, or other characteristics in products must be justified (Abedi-Firoozjah et al., 2024). To achieve these goals, manufacturers have turned to a wide range of different substances—natural spices, herbs, and extracts, as well as synthetic additives. Animal- and plant-based powder proteins are also emerging options that improve the functionality of meat products while contributing to a boost in protein intake (Goemaere et al., 2021). The diversity of additives added to foods to target specific

desirable effects highlights the growing importance of the additive industry, where more attention is given to additives than to the product itself (Prica et al., 2012).

When appropriately used, food additives help preserve quality and safety, extend shelf life, and improve the sensory properties of the products. However, excessive and inappropriate use of additives can negatively affect not only the quality of the product but also the health of consumers (Willett, 2024). Regular market monitoring and periodic safety re-evaluation of additives used in meat products industry is crucial to ensure compliance with established limits and standards. This approach helps build trust in the food chain and aligns with consumer preferences, while industry innovations increasingly favor natural alternatives. Furthermore, regular monitoring and re-evaluation of quantity and safety assessments of additives used in meat products are used to ensure that they meet certain standards and requirements and, finally, to establish trust in the food chain and align with consumer preferences (Abedi-Firoozjah et al., 2024). According to the Rulebook on food additives (2018), all ingredients, including additives, must be clearly listed on the product label, and their labeling is by specific regulations on declaration, labelling and advertising on food, Rulebook (2024).

The addition of various additives, such as phosphates, is aligned with modern technological standards from developed European countries. Phosphates stand out as one of the fundamental additives in meat products industry due to their functional properties as emulsifiers, stabilizers, and acid regulators influencing the pH value of the products. Regardless of the primary mechanism of action, their basic function is to increase the ability of meat to retain its own and added water, thereby improving the texture, juiciness, and tenderness of the product (Saičić et al., 2008; Đerić et al., 2015; Cao et al., 2022). Phosphates are commonly used in products such as cooked sausages and smoked meat products. The most commonly used are disodium and potassium diphosphates, pyrophosphates, and polyphosphates, usually in quantities of 0.3-0.5% per kilogram of meat, which increases water retention capacity by 5-10% and increases the meat yield by about 2-3% (Đerić et al., 2015).

Although phosphates hold an important technological role, phosphorus as an element also has vital biological functions for both human and animal organisms, with 85% of the total phosphorus found in bones and teeth (Prica et al., 2015). However, excessive phosphorus intake can lead to health problems, as the imbalance between calcium and phosphorus can disrupt hormonal processes. For example, excess phosphorus can interfere with calcium absorption in the intestines, causing calcium release from bones and increasing the risk of bone diseases (Đerić et al., 2015; Cao et al., 2022).

It is important to note that meat, as a raw material, naturally contains phosphorus in concentrations that may vary depending on the animal species, the specific part of the carcass, the diet, and the processing methodology used.

However, the excessive addition of synthetic phosphates can increase water retention, diminishing the product's nutritional value (Perši et al., 2010). The balanced use of phosphates should ensure the desired product quality while minimizing potential health risks due to excessive phosphorus intake.

Changes in consumer awareness and their preference for natural and health-friendly food products have led to the widespread adoption of the “clean label” initiative among many food industry participants (Asioli et al., 2017). Although a legal definition or specific regulations for “clean label” products have not yet been established, consumer demand for meat products with natural ingredients has steadily increased in recent years (Câmara et al., 2020). According to the research performed by Maruyama et al. (2021), consumers view stabilizers and thickeners as unnatural and less acceptable in food formulations, suggesting that companies wishing to produce products should cease using or replace these ingredients. Thus, the meat industry faces a significant challenge in replacing phosphates with natural ingredients without compromising product quality.

This study aimed to determine the total phosphorus content in meat products available on the market of the Republic of Serbia and assess producers' compliance with the applicable legal regulations regarding the allowable amounts of additives.

Materials and Methods

During a two-year period (2022–2024), 74 meat products collected from the territory of Belgrade were analyzed with the aim of determining the total phosphorus content, expressed as P_2O_5. Of the total number, 41 samples consisted of finely minced cooked sausages (36 samples of small diameter and 5 samples of large diameter), while the remaining 33 samples were coarsely minced cooked sausages. Each product was analyzed in three replicates based on three different lot numbers, totaling 222 samples. The analysis was conducted using the standard ISO method (SRPS ISO 13730:1999) at the Institute of Animal Husbandry, Belgrade – Zemun laboratory. Absorbance was measured using a UV/VIS spectrophotometer (Analytik Jena, SPEKOL 1300). The results are expressed in g/kg as P_2O_5. Upon receiving them in the laboratory, samples were stored in their original packaging at a temperature of 4°C until analysis, or, in cases where they were not vacuum-sealed, homogenized and analyzed within 24 hours after homogenization.

Data processing was carried out using MS Excel. The results are presented as the mean content of total phosphorus, expressed as P_2O_5 (g/kg) ± standard deviation.

Results and Discussion

The total phosphorus content, expressed as P_2O_5 (g/kg) in meat products is presented in tables 1 and 2. Table 1 provides data for finely minced cooked sausages, with a range of total phosphorus content from 2.01 g/kg to 6.98 g/kg, as P_2O_5, while Table 2 shows the total phosphorus content in coarsely minced cooked sausages, ranging from 4.13 g/kg to 7.97 g/kg, as P_2O_5. The results demonstrated that all samples had total phosphorus content within the permissible limits according to the Rulebook on quality of chopped meat, semi-products and products of meat (2023), i.e., below 8 g/kg. The average content of total phosphorus in all analyzed finely minced cooked sausages and coarsely minced cooked sausages was 4.63 g/kg and 6.04 g/kg, respectively.

Table 1. Content of total phosphorus, expressed as P_2O_5 (g/kg) in finely minced cooked sausages

Product type (sausage type)	Average content of total phosphorus Mean±SD (g/kg)	CV (%)	Min (g/kg)	Max (g/kg)
	Small-diameter finely minced cooked sausages, n=36			
Hot dog sausage, n=6	4.17±1.04	0.25	2.82	5.79
Hot dog-style sausage, n=27	4.88±1.13	0.23	2.01	6.98
Debreciner sausage, n=3	3.76±0.56	0.15	3.05	4.72
	Large-diameter finely minced cooked sausages, n=5			
Extra sausage, n=5	4.34±0.67	0.16	3.39	5.49
Total	4.63±1.12	0.24	2.01	6.98

n − total number of analysed samples; SD - standard deviation; CV - coefficient of variation; Min – minimum; Max - maximum

In the study by Milicević et al. (2021), the average phosphate content in finely minced cooked sausages was 4.68 g/kg (total phosphorus as P_2O_5). Other authors report similar results: Petrović (2022) found an average value of 4.83 g/kg, Korićanac et al. (2015) reported 4.89 g/kg, while Saičić (2008) noted a somewhat lower value (2.13 g/kg). These findings align with the results of our study. Milešević et al. (2022) also investigated the phosphate content in various meat products. According to their results, the average total phosphorus content in finely minced cooked sausages was 4.70 g/kg, while coarsely minced cooked sausages had a slightly higher average value of 5.21 g/kg. The highest values for total phosphorus were recorded in smoked meat products, while the lowest were found in liver sausage and pâté. In addition to phosphate content, these authors assessed

phosphorus intake through processed meat products, particularly in children. Their research showed that the daily phosphorus intake from processed meat products in children was significantly below the recommended permissible values, with the primary sources of phosphorus being cooked sausages, canned meat, and bacon. Considering all dietary sources of phosphorus, including bakery products, cheeses, and sugars, there is a possibility of exceeding the recommended daily intake (EFSA, 2019).

Table 2. Content of total phosphorus, expressed as P_2O_5 (g/kg) in coarsely minced cooked sausages

Product type (sausage type)	Average content of total phosphorus Mean±SD (g/kg)	CV	Min (g/kg)	Max (g/kg)
	Minced cooked sausages n=33			
Beef sausage, n=4	6.06±0.61	0.10	5.23	7.38
Novosadska sausage, n=4	6.20±0.43	0.07	5.43	6.81
Grill sausage, n=6	6.71±0.60	0.09	5.31	7.60
Srpska sausage, n=4	5.97±0.44	0.07	5.39	6.85
Domaća sausage, n=4	6.07±1.23	0.20	4.13	7.97
Tirolska sausage, n=7	5.19±0.51	0.10	4.50	6.41
Toast sausage, n=4	6.34±0.75	0.12	5.30	7.87
Total	6.04±0.83	0.14	4.13	7.97

n − total number of analysed samples; SD - standard deviation; CV - coefficient of variation; Min – minimum; Max - maximum

In contrast to our study, where all analized samples had total phosphorus content below allowable value (8g/kg, as P_2O_5), Milešević et al. (2022) found that 1.7% of the analyzed meat products had phosphate levels exceeding 8 g/kg. Similarly, Milicević et al. (2021) reported that 0.5% of the samples exceeded the permissible limit, while Korićanac et al. (2015) found that 0.43% of the products did not meet the defined limits. These results imply the rare misuse of phosphates in meat processing, resulting in excess allowable levels in some samples. Such irregularities underline the importance of continued monitoring, joint measures, and actions to enforce phosphate regulations, assuring compliance and protecting consumer safety.

Conclusions

All analyzed samples were within the permissible total phosphorus. The average total phosphorus content expressed as P_2O_5 (g/kg) was 4.63 g/kg for finely minced sausages and 6.04 g/kg for coarsely minced sausages. However, analytical methods cannot distinguish between naturally occurring phosphates and food additives. Interpreting these results requires an understanding of natural phosphorus levels in raw materials. Nevertheless, monitoring phosphate additives in meat products to ensure compliance with acceptable limits is essential, as this is critical for consumer health and product quality and safety. Based on the comprehensive market survey, it can be concluded that producers are using phosphates appropriately, in accordance with the guidelines outlined in the Rulebook (2023), despite variations in meat product preparation technologies.

Sadržaj ukupnog fosfora u proizvodima od mesa na tržištu Republike Srbije i usklađenost sa propisima

Maja Petričević, Nikola Stanišić, Tanja Keškić,Veselin Petričević, Dragan Nikšić, Boris Pisinov, Tamara Stamenić

Rezime

Ovo istraživanje se fokusiralo na praćenje nivoa fosfata u proizvodima od mesa na tržištu Srbije u periodu od dve godine i procenu usklađenosti proizvođača sa propisima o upotrebi aditiva. Tokom pomenutog perioda analizirana su 74 različita proizvoda od mesa (ukupno 222 uzorka), uključujući fino i grubo usitnjene barene kobasice. Analiza je sprovedena standardnom metodom (SRPS ISO 13730:1999) u Laboratoriji Instituta za stočarstvo, Beograd – Zemun. Sadržaj ukupnog fosfora, izražen kao P_2O_5 se kretao od 2,01 g/kg do 6,98 g/kg kod fino usitnjenih kobasica, dok je kod grubo usitnjenih bio između 4,13 g/kg i 7,97 g/kg. Rezultati pokazuju da proizvođači koriste fosfate u skladu sa propisanim granicama.

Ključne reči: aditivi, ukupan fosfor, fosfati, proizvodi od mesa, Srbija

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Conflict of interest
The authors declare that they have no conflict of interest.

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