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EFFECT OF UDDER HEALTH STATUS ON DAILY MILK PRODUCTION PARAMETERS CONSIDERING ANIMAL-RELATED FACTORS IN HOLSTEIN CATTLE

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Abstract: This study analysed the effect of udder health status on daily milk production parameters in Holstein cattle, considering lactation stage, parity, and production level. A total of 2,509,222 test-day records were analyzed, with somatic cell count (SCC) used as a key indicator for detecting mastitis. Cows were classified into three health categories based on SCC thresholds: healthy (<200,000 cells/ml), subclinical mastitis (200,000–400,000 cells/ml), and clinical mastitis (>400,000 cells/ml). The results showed a significant reduction in daily milk yield in cows affected by mastitis, with the most pronounced declines observed in clinical cases. Additionally, milk composition parameters were influenced by udder health status, with fat and protein content increasing, while lactose concentration decreased in mastitic cows. Across all production levels and parity classes, mastitic cows exhibited lower milk urea concentrations, suggesting potential metabolic alterations. Obtained results highlight the detrimental impact of mastitis on milk production efficiency and composition, reinforcing the need for effective disease prevention and management strategies. Precision monitoring and early mastitis detection remain crucial for optimizing milk quality, improving dairy farm sustainability, and ensuring animal welfare.

Key words: mastitis, daily milk production, somatic cell count, Holstein cattle

Introduction

Production diseases in dairy cows primarily stem from management practices, particularly inadequate feeding and handling. These include metabolic

disorders, infertility, mastitis, and laminitis, where nutritional and managerial factors play a key role (Nir, 2003). While infectious diseases attract more public attention, production diseases have a far greater economic impact, significantly exceeding that of epidemic outbreaks (Hogeveen et al., 2019). Addressing these issues through improved management enhances animal health and the economic efficiency of dairy production. Mastitis is the most prevalent production disease in dairy cattle (Seegers et al., 2003), primarily caused by intramammary infections (IMI) from bacterial pathogens. It leads to physical, chemical, and bacteriological changes in milk, as well as pathological alterations in gland tissue. Mastitis occurs in clinical and subclinical forms. Clinical mastitis presents with visible symptoms such as milk abnormalities, gland inflammation, and systemic illness, while subclinical mastitis is asymptomatic and detected via elevated somatic cell count (SCC) (Adkins and Middleton, 2018). Based on severity, clinical mastitis is classified as mild (milk changes), moderate (gland inflammation), and severe (systemic symptoms) (Narváez-Semanate et al., 2022). Mild cases are most common, with severe cases being rare. Furthermore, mastitis significantly affects milk production, quality, and farm economics, emphasizing the need for early detection and treatment.

Mastitis diagnosis relies on clinical observations and inflammatory response measurements, while identifying the causative agent is crucial for diagnosing IMI. Subclinical mastitis, more prevalent than clinical forms, accounts for up to 80% of milk production losses and poses a greater long-term risk (Halasa et al., 2007). If untreated, affected udder quarters may dry out, leading to culling or death. Despite its economic impact, preventive measures are often delayed until milk yield declines. Early detection is essential, as udder tissue changes occur before visible symptoms (Argaw, 2016). Timely diagnosis reduces antibiotic use, preserves milk production, and prevents infection spread (Kamal et al., 2014). Additionally, mastitis contributes to greenhouse gas emissions, making prevention critical for both sustainability and profitability (Özkan Gülzari et al., 2018). Furthermore, effective management minimizes losses, optimizes culling rates, and reduces emissions per kilogram of milk produced.

SCC represents a key indicator of intramammary infections and milk quality, monitored at individual, herd, and population levels (Schukken et al., 2003). Tracking SCC provides insights into udder health, as elevated counts typically indicate inflammation due to pathogen invasion, which triggers an immune response and increases SCC in milk (Ivanov et al., 2016; Alhussien and Dang, 2018). An increase in SCC during successive lactations is primarily linked to polymorphonuclear leukocytes (PMN), while SCC fluctuations within lactations involve both PMN and other immune cells (Blackburn, 1966). Several factors influence SCC variability, including lactation stage, parity, season, management practices, and environmental conditions. SCC tends to increase with lactation number due to accumulated udder stress and immune system changes (Mikone et

al., 2016). Elevated SCC levels in late-lactation cows are often associated with reduced milk outflow and a decline in udder immune defence mechanisms. Heat stress exacerbates SCC fluctuations, as shown in studies on Holstein cows, where SCC levels varied with temperature-humidity index, milk yield, breed, and parity (Gantner et al., 2011; Gantner et al., 2017). Furthermore, herd management plays a crucial role in SCC control. Poor milking hygiene, improper equipment maintenance, and inadequate housing conditions contribute to bacterial infections and elevated SCC (Hadrich et al., 2018). Healthy udder function is associated with SCC levels below 100,000 cells/ml, with optimal milk quality ranging between 20,000 and 100,000 SCC/ml (Mikone et al., 2016). Elevated SCC leads to significant production losses, exceeding 8% in cows with SCC levels between 50,000 and 100,000 cells/ml, and reaching 15–18% in those with SCC levels from 100,000 to 250,000 cells/ml (Pfützner and Ózsvári, 2016). The overall impact on milk yield depends on SCC distribution across cows and parity within the herd (Chen et al., 2023). Finally, managing SCC effectively through improved hygiene, nutrition, and environmental control is essential for maintaining milk production efficiency and reducing economic losses.

Considering the importance of mastitis analysis on dairy cattle farms, this study aimed to analyse the effect of udder health status (healthy, subclinical or clinical mastitis) on daily milk production parameters in Holstein cattle, considering key animal-related factors (lactation stage, parity and animal production level).

Materials and Methods

The statistical analysis in this research was based on test-day records from Holstein cows reared on dairy cattle farms in Croatia, collected over a 10-year period (January 2013–December 2022) as part of routine milk recording. The milk recording process adhered to the standards established by the International Committee for Animal Recording (ICAR, 2017) for alternative milk recording methods (AT4/BT4). Under this system, milk yield measurements and sample collection were conducted every four weeks, with samples obtained from each cow during either the morning or evening milking. Data collection for the AT4 method was carried out by trained officers from the Croatian Agency for Agriculture and Food (HAPIH), while farm employees trained in milk recording performed the BT4 method. After collection, milk samples were transported to the accredited Central Laboratory for Milk Quality Control (SLKM) of HAPIH for analysis, ensuring full compliance with ICAR (2017) protocols.

Milk composition analysis included fat, protein, lactose, total solids, solids-not-fat, urea, casein, free fatty acids, pH, ketone bodies, and freezing point. These parameters were determined using infrared spectrophotometry on MilkoScan

analyzers. Additionally, somatic cell counts were measured using Fossomatic analyzers, employing the fluoro-opto-electronic method.

Before implementing logical data control procedures, the dataset obtained from HAPIH contained over 4 million test-day records. During the validation process, entries that did not meet the standards set by ICAR (2017) were excluded. Additionally, records with missing or inaccurate details regarding breed, farm identification, breeding region, lactation stage, parity, age at first calving, calving date, and milk recording date were removed to ensure data integrity and consistency. After applying these quality control measures, the final dataset comprised 2,509,222 test-day records from Holstein cows.

Somatic cell count (SCC) was used as a primary indicator for identifying subclinical and clinical mastitis. Based on SCC levels, cows were classified into three categories: healthy (<200,000 cells/ml), subclinical mastitis (200,000–400,000 cells/ml), and clinical mastitis (>400,000 cells/ml). To account for the effect of the lactation stage, cows were categorized into four days-in-milk (DIM) classes: < 100 days, 100–200 days, 200–300 days, and > 300 days. Furthermore, regarding the parity animals were classified into four groups: first (I), second (II), third (III), and fourth or higher (≥ IV). Additionally, cows were divided into four groups based on their daily production level (I. DMY < 20 kg; II. DMY in 20 – 30 kg; III. DMY in 30 – 40 kg; IV. DMY > 40 kg).

The analysis of the effect of udder health status (healthy, subclinical or clinical mastitis) on daily milk production parameters in Holstein cattle was analysed separately by the classes of lactation stage, parity and animal production level using the following statistical model:

$$y_{ijklmn} = \mu + b_1\left(\frac{d_i}{305}\right) + b_2\left(\frac{d_i}{305}\right)^2 + b_3\, ln\left(\frac{305}{d_i}\right) + b_4\, ln^2\left(\frac{305}{d_i}\right)$$

$$+A_j + R_k + H_l + S_m + M_n + e_{ijklmn}$$

where:

y_{ijklmn} = estimated milk production trait (daily milk yield, daily fat, protein, lactose and urea content, log value of somatic cell count);

μ = intercept;

b_1, b_2, b_3, b_4 = regression coefficients;

d_i = stage of lactation (i = 6 to 400 day);

A_j = fixed effect of age at first calving (j = 21 to 36 month) * only for firs parity;

R_k = fixed effect of region k (k = Central, Eastern, Mediterranean);

H_l = fixed effect of herd size (l = I, II, III, IV, V, VI);

S_m = fixed effect of season (m = spring, summer, autumn, winter);

M_n = fixed effect of udder health status M (n = healthy, subclinical mastitis, and clinical mastitis);

e_{ijklmn} = residual.
The significance of the differences between the estimated LSMeans was tested by Scheffe's method of multiple comparisons using the MIXED procedure of SAS (SAS Institute Inc., 2019).

Results and Discussion

The results in Table 1 show the effect of udder health status on daily milk production parameters across different lactation stages. Healthy cows consistently exhibited the highest daily milk yield, while cows with subclinical and clinical mastitis showed significantly lower values. This decline was statistically significant, indicating that mastitis negatively affects milk production. Milk composition also varied depending on health status. Both fat and protein content were higher in cows with clinical mastitis than in healthy cows, suggesting that inflammation-related changes influence milk synthesis. In contrast, lactose content was notably lower in cows with mastitis, particularly in clinical cases, which reflects impaired udder function and reduced milk quality. Somatic cell count, a key indicator of udder health, was significantly elevated in cows with mastitis, with the highest values recorded in the clinical group.

Table 1. LsMeans of daily milk production parameters regarding the udder health status and considering lactation stage classes (< 100 days, 100 – 200 days, 200 – 300 days, > 300 days)

Lactation stage class	Health status	DMY	DFC	DPC	DLC	SCClog	UREA
< 100 days	Healthy	25.72^{A}	3.96^{A}	3.12^{A}	4.54^{A}	5.61^{A}	21.35^{A}
	Subclinical	25.45^{B}	4.05^{B}	3.17^{B}	4.48^{B}	8.12^{B}	20.77^{B}
	Clinical	25.11^{C}	4.09^{C}	3.20^{C}	4.40^{C}	10.18^{C}	20.50^{C}
100 – 200 days	Healthy	24.93^{A}	3.97^{A}	3.33^{A}	4.53^{A}	5.81^{A}	22.65^{A}
	Subclinical	24.74^{B}	4.02^{B}	3.36^{B}	4.47^{B}	8.10^{B}	22.16^{B}
	Clinical	24.58^{C}	4.02^{B}	3.39^{C}	4.39^{C}	10.14^{C}	22.02^{C}
200 – 300 days	Healthy	24.12^{A}	4.11^{A}	3.52^{A}	4.50^{A}	6.05^{A}	22.52^{A}
	Subclinical	23.93^{B}	4.15^{B}	3.54^{B}	4.44^{B}	8.05^{B}	22.16^{Bb}
	Clinical	23.85^{C}	4.14^{B}	3.57^{C}	4.36^{C}	10.00^{C}	22.06^{Bc}
> 300 days	Healthy	23.75^{A}	4.21^{A}	3.67^{A}	4.48^{A}	6.23^{A}	22.27^{Aa}
	Subclinical	23.47^{B}	4.23^{B}	3.69^{B}	4.41^{B}	8.02^{B}	22.16^{b}
	Clinical	23.35^{C}	4.22^{B}	3.71^{C}	4.33^{C}	9.86^{C}	22.06^{Bb}

*LsMeans marked with different letter (capital or small), differ statistically highly ($p < 0.001$) significant or statistically ($p < 0.01$) significant
DMY – daily milk yield (kg), DFC – daily fat content (%), DPC – daily protein content (%), DLC – daily lactose content (%), SCClog – log value of somatic cell count, UREA – concentration of urea in milk (mg/dl)

This confirms that increased SCC is closely associated with mastitis severity and inflammation. Milk urea content showed smaller yet significant differences between health statuses. Healthy cows generally had slightly higher urea values, whereas cows with mastitis exhibited lower levels, possibly due to changes in nitrogen metabolism caused by udder inflammation. Finally, mastitis significantly impacts both milk yield and composition, reducing daily production, altering fat, protein, and lactose levels, and leading to a substantial increase in somatic cell count.

The results in Table 2 show the effect of udder health status on daily milk production parameters across different parity classes (I, II, III, and VI+). Healthy cows consistently had the highest milk production. In parity I, healthy cows produced an average of 24.13 kg of milk per day, which was significantly higher compared to subclinical (23.99 kg) and clinical cows (23.91 kg). This trend of decreasing milk yield with increasing severity of mastitis was observed across all parity classes, with clinical cows showing the lowest daily milk yields. Regarding daily fat content (DFC), there was a slight but significant increase in fat content as mastitis severity worsened. In the first parity class, healthy cows had a fat content of 4.02%, which increased to 4.12% in clinical cows. This trend was consistent across all parity classes, indicating that mastitis may slightly increase fat content in milk, though the effect was not large. The daily protein content (DPC) followed a similar pattern, where protein levels were slightly higher in cows with subclinical and clinical mastitis than in healthy cows. For instance, in the first parity class, Healthy cows had a protein content of 3.37%, while subclinical cows had 3.40%, and clinical cows had 3.43%. This suggests that mastitis severity may slightly influence protein content in milk, although the differences were not very pronounced.

Daily lactose content (DLC) showed a consistent trend, with clinical mastitis cows having lower lactose levels compared to Healthy cows. In the first parity class, Healthy cows had a lactose content of 4.58%, while clinical cows had 4.46%. This decline in lactose content with increasing mastitis severity was consistent across all parity classes, highlighting a potential negative impact of mastitis on lactose synthesis. Somatic cell count (SCClog), represented as the logarithmic value, showed a clear increase with the severity of mastitis. In the first parity class, Healthy cows had a SCClog of 5.71, which increased to 9.95 in clinical cows. This indicates that somatic cell count, a marker of inflammation and infection, rises significantly with mastitis severity. The trend was consistent across all other parity classes, where clinical cows always exhibited the highest SCClog values. Finally, urea concentration (UREA) in milk was generally lower in cows with clinical mastitis compared to Healthy cows. For example, in the first parity class, Healthy cows had a urea concentration of 22.11 mg/dl, while clinical cows had a slightly lower value of 21.86 mg/dl. This reduction in urea concentration with worsening mastitis severity was observed across all parity classes.

Table 2. LsMeans of daily milk production parameters regarding the udder health status and considering parity classes (I, II, III, and VI+)

Parity class	Mastitis class	DMY	DFC	DPC	DLC	SCClog	UREA
I	Healthy	24.13^{A}	4.02^{A}	3.37^{A}	4.58^{A}	5.71^{A}	22.11^{A}
	Subclinical	23.99^{B}	4.10^{B}	3.40^{B}	4.53^{B}	8.02^{B}	21.75^{Bb}
	Clinical	23.91^{C}	4.12^{C}	3.43^{C}	4.46^{C}	9.95^{C}	21.86^{Bc}
II	Healthy	24.06^{A}	4.08^{A}	3.42^{A}	4.52^{A}	5.83^{A}	22.21^{A}
	Subclinical	24.79^{B}	4.12^{B}	3.46^{B}	4.46^{B}	8.02^{B}	21.77^{Bb}
	Clinical	24.60^{C}	4.13^{C}	3.48^{C}	4.39^{C}	10.02^{C}	21.66^{Bc}
III	Healthy	25.24^{A}	4.09^{A}	3.38^{A}	$4,49^{A}$	5.96^{A}	22.14^{A}
	Subclinical	24.97^{B}	4.12^{B}	3.42^{B}	4.43^{B}	8.06^{B}	21.65^{B}
	Clinical	24.79^{C}	4.13^{B}	3.44^{C}	4.35^{C}	10.09^{C}	21.44^{C}
VI+	Healthy	25.08^{A}	4.05^{A}	3.34^{A}	4.47^{A}	6.04^{A}	22.17^{A}
	Subclinical	24.81^{B}	4.08^{B}	3.38^{B}	$4,41^{B}$	8.08^{B}	21.69^{B}
	Clinical	24.63^{C}	4.09^{C}	3.41^{C}	4.31^{C}	10.16^{C}	21.38^{C}

*LsMeans marked with different letter (capital or small), differ statistically highly ($p < 0.001$) significant or statistically ($p < 0.01$) significant;
DMY – daily milk yield (kg), DFC – daily fat content (%), DPC – daily protein content (%), DLC – daily lactose content (%), SCClog – log value of somatic cell count, UREA – concentration of urea in milk (mg/dl)

The results presented in Table 3 show the effect of udder health status on daily milk production parameters across different production levels (I, II, III, and VI class). In all production classes, cows classified as healthy (normal group) exhibited significantly higher daily milk yield (DMY) compared to those affected by subclinical and clinical mastitis. The greatest reduction in milk production was observed in cows with clinical mastitis, with the most pronounced difference found in the highest production class (VI.), where healthy cows produced an average of 38.21 kg per day, whereas those with clinical mastitis produced 37.47 kg.

Milk fat content (DFC) showed a slight increase with the severity of mastitis. In the first production class, healthy cows had a fat content of 4.27%, whereas those with clinical mastitis exhibited a slightly higher percentage (4.36%). This trend was consistent across all production classes, suggesting that mastitis may lead to an increase in milk fat content, likely due to changes in milk composition resulting from the inflammatory response. Milk protein content (DPC) also increased with the severity of mastitis, with cows suffering from clinical mastitis showing the highest values. For instance, in production class I., healthy cows had a protein content of 3.54%, while those with clinical mastitis had 3.64%. This pattern indicates alterations in milk protein composition in infected animals,

potentially due to an increased presence of immune-related proteins in response to inflammation.

Lactose content (DLC) showed a declining trend as mastitis severity increased. Healthy cows in all production classes had significantly higher lactose concentrations compared to those with subclinical and clinical mastitis. In production class I., healthy cows exhibited an average lactose content of 4.43%, while cows with clinical mastitis had a lower concentration of 4.24%. This decline was even more pronounced in higher production classes, indicating a reduced ability to synthesize lactose in the presence of inflammation in the mammary gland. The log-transformed somatic cell count (SCClog) values significantly increased with the severity of mastitis, confirming the strong association between mastitis and elevated somatic cell counts in milk. In all production classes, healthy cows exhibited the lowest SCClog values, whereas cows with clinical mastitis had the highest values. For example, in production class VI, SCClog was 5.53 in healthy cows, whereas it reached 10.00 in cows with clinical mastitis. This increase reflects the strong inflammatory response triggered by mastitis, leading to a significant influx of somatic cells into the milk.

Milk urea concentration (UREA) was slightly lower in cows affected by mastitis compared to healthy cows, although the differences were less pronounced than for other parameters. In the first production class, healthy cows had an average urea concentration of 20.86 mg/dl, while those with clinical mastitis had a slightly lower value of 20.62 mg/dl. A similar trend was observed across higher production classes, where healthy cows consistently showed slightly higher urea concentrations compared to those with subclinical and clinical mastitis.

Table 3. LsMeans of daily milk production parameters regarding the udder health status and considering animal production level (I, II, III, and VI+)

Production class	Mastitis class	DMY	DFC	DPC	DLC	SCClog	UREA
I DMY < 16 kg	**Healthy**	12.44^{A}	4.27^{A}	3.54^{A}	4.43^{A}	6.26^{A}	20.86^{A}
	Subclinical	12.07^{B}	4.34^{B}	3.59^{B}	4.34^{B}	8.11^{B}	20.66^{B}
	Clinical	11.92^{C}	4.36^{C}	3.64^{C}	4.24^{C}	10.14^{C}	20.62^{B}
II DMY in 16 – 24 kg	**Healthy**	20.07^{A}	4.14^{A}	3.46^{A}	4.50^{A}	6.02^{A}	21.62^{A}
	Subclinical	19.91^{B}	4.18^{B}	3.49^{B}	4.44^{B}	8.08^{B}	21.32^{Bb}
	Clinical	19.86^{C}	4.18^{B}	3.52^{C}	4.36^{C}	10.11^{C}	21.22^{Bc}
III DMY in 24 – 32 kg	**Healthy**	27.71^{A}	3.99^{A}	3.36^{A}	4.54^{A}	5.81^{A}	22.56^{A}
	Subclinical	27.59^{B}	4.03^{Bb}	3.39^{B}	4.49^{B}	8.06^{B}	22.12^{B}
	Clinical	27.54^{C}	4.04^{Bc}	3.41^{C}	4.42^{C}	10.06^{C}	21.95^{C}
VI DMY > 32 kg	**Healthy**	38.21^{A}	3.84^{A}	3.21^{A}	4.58^{A}	5.53^{A}	23.31^{A}
	Subclinical	37.93^{B}	3.90^{B}	3.24^{B}	4.53^{B}	8.06^{B}	22.72^{B}
	Clinical	37.47^{C}	3.92^{C}	3.26^{C}	4.46^{A}	10.00^{C}	22.30^{C}

*LsMeans marked with different letter (capital or small), differ statistically highly ($p < 0.001$) significant or statistically ($p < 0.01$) significant;
DMY – daily milk yield (kg), DFC – daily fat content (%), DPC – daily protein content (%), DLC – daily lactose content (%), SCClog – log value of somatic cell count, UREA – concentration of urea in milk (mg/dl)

The results of this study are consistent with previous findings indicating that mastitis prevalence negatively impacts milk production and quality. Schukken et al. (2003) emphasized the importance of somatic cell count (SCC) as a diagnostic tool for assessing udder health, and our findings confirm that elevated SCC is a strong indicator of infection, particularly in clinical cases. Elevated SCC values were significantly higher in cows with mastitis, particularly in the clinical health status group, reflecting the inflammatory response to infection (Alhussien and Dang, 2018). This increase in SCC correlates with a decrease in milk yield, which is consistent with the findings of Mikone et al. (2016) and Hadrich et al. (2018), who reported that higher SCC levels are associated with reduced milk production. Moreover, the changes in milk composition observed in this study, higher fat and protein levels in cows with clinical mastitis, are in line with findings from Blackburn (1966), who noted that inflammatory processes can alter milk synthesis. The reduction in lactose content, particularly in cows with clinical mastitis, aligns with previous studies showing that mastitis often leads to decreased milk quality due to impaired lactose synthesis (Smith et al., 2001). The impact of mastitis on urea content in milk, though less pronounced, also supports findings by Mikone et al. (2016), who noted slight variations in urea levels associated with udder health status. The lower urea values in mastitic cows may reflect changes in nitrogen metabolism due to infection and inflammation (Alhussien and Dang, 2018). These findings underline the economic significance of mastitis, as cows with subclinical and clinical mastitis experience not only reduced milk yield but also changes in milk composition that affect quality. Finally, as emphasized by Pfützner and Ózsvári (2016), early detection and management of mastitis can mitigate these losses, highlighting the need for effective monitoring and intervention strategies in dairy herds.

Conclusion

The obtained results, indicate the significant impact of udder health status on daily milk production parameters in Holstein cattle, considering lactation stage, parity, and production level. Across all analyzed factors, cows diagnosed with subclinical and clinical mastitis exhibited lower daily milk yield compared to their healthy counterparts, with the greatest reductions observed in animals suffering from clinical mastitis. Additionally, milk composition was notably affected, with

mastitis-associated increases in milk fat and protein content, while lactose concentration consistently declined as the severity of mastitis increased.

Somatic cell count, a key indicator of mastitis, reaffirms its role as a crucial parameter for monitoring udder health. The negative effects of mastitis, indicated by the SCC, on milk production traits were evident regardless of lactation stage, parity, or production level, indicating that disease management strategies should be implemented comprehensively across all groups of dairy cows. Furthermore, minor but consistent reductions in milk urea concentration were observed in cows with the highest SCC, suggesting potential alterations in nitrogen metabolism related to udder health status. Finally, the obtained results emphasize the critical need for effective mastitis prevention and control strategies to maintain optimal milk production and composition in dairy herds. Considering the economic and physiological consequences of mastitis, precision health monitoring and early detection remain essential for improving overall dairy farm efficiency and animal welfare.

Uticaj zdravstvenog stanja vimena na dnevne parametre proizvodnje mleka uzimajući u obzir faktore povezane sa životinjama kod holštajnskih krava

Boris Ljubojević, Zvonimir Steiner, Čedomir Radović, Ranko Gantner, Klemen Potočnik, Vesna Gantner

Rezime

Ova studija je analizirala uticaj zdravstvenog statusa vimena na parametre dnevne proizvodnje mleka kod holštajnskih krava, uzimajući u obzir fazu laktacije, redosled teljenja i nivo proizvodnje. Ukupno je analizirano 2.509.222 zapisa sa kontrolnih muži, pri čemu je broj somatskih ćelija (SCC) korišćen kao ključni pokazatelj za detekciju mastitisa. Krave su klasifikovane u tri zdravstvene kategorije na osnovu SCC pragova: zdrave (<200.000 ćelija/ml), subklinički mastitis (200.000–400.000 ćelija/ml) i klinički mastitis (>400.000 ćelija/ml). Rezultati su pokazali značajno smanjenje dnevnog prinosa mleka kod krava obolelih od mastitisa, pri čemu su najizraženiji padovi zabeleženi u slučajevima kliničkog mastitisa. Takođe, zdravstveni status vimena uticao je na parametre sastava mleka – zabeleženo je povećanje sadržaja masti i proteina, dok je koncentracija laktoze bila smanjena kod krava sa mastitisom. Kod svih nivoa proizvodnje i redosleda teljenja, krave sa mastitisom su imale nižu koncentraciju uree u mleku, što ukazuje na moguće metaboličke promene. Dobijeni rezultati ističu štetan uticaj mastitisa na efikasnost proizvodnje mleka i njegov sastav,

naglašavajući potrebu za efikasnim strategijama prevencije i upravljanja bolešću. Precizno praćenje i rana detekcija mastitisa ostaju ključni za optimizaciju kvaliteta mleka, unapređenje održivosti mlečne proizvodnje i očuvanje dobrobiti životinja.

Ključne reči: mastitis, dnevna proizvodnja mleka, broj somatskih ćelija, holštajnske krave

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

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CORRELATION BETWEEN EJACULATE CHARACTERISTICS AND SPERM ABNORMALITIES IN BOARS EXPOSED TO SUMMER HEAT STRESS

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Abstract: Seasonal heat stress is a well-known factor that negatively affects boar fertility, primarily by reducing semen quality and increasing the incidence of abnormal spermatozoa. This study aimed to investigate the relationship between ejaculate characteristics and sperm morphological abnormalities in boars during the most sensitive period of the year. Between August and October, a total of 129 ejaculates were collected from 17 boars. The analyzed semen traits included ejaculate volume, sperm concentration, total and functional sperm counts, motility (in native and diluted semen), viability, and the presence of morphological abnormalities, specifically head, tail, acrosomal defects, and protoplasmic droplets. The results showed that only sperm motility and the number of produced doses significantly correlated with abnormal sperm forms. A weak but consistent positive correlation (r_f>0.200; $p<0.05$ or $p<0.01$) was observed between certain categories of abnormalities, particularly between secondary forms (such as protoplasmic droplets) and primary defects in the tail and acrosomal region. Correlations among primary abnormalities were generally weak. These findings highlight the importance of monitoring sperm motility and morphology as early indicators of heat stress effects in boars.

Key words: boar, semen quality, sperm abnormalities, ejaculate traits, heat stress

Introduction

Given that artificial insemination allows for the production of a large number of offspring from a single boar (Knecht et al., 2014; Apić et al., 2016),

evaluating sperm morphology is a key component of animal breeding programs, as it supports the selection of genetically and reproductively superior males (Gadea, 2025). Routine semen assessments are primarily effective in detecting clear cases of infertility, and in some instances, may also suggest early signs of reduced fertility (Barna et al., 2021). High-quality boar semen plays a crucial role in the success of artificial insemination (Bonet et al, 1993; Li et al. 2023).

The success of AI largely depends on the quantitative and qualitative traits of the ejaculate (Savić et al., 2015). Therefore, the evaluation of boar ejaculate quality represents the first and essential step in semen processing, to ensure that only ejaculates capable of producing quality insemination doses are selected for further use (Rozeboom, 2000). To get the best reproductive performance from each boar, it's important to consider genetic traits and external factors influencing semen quality and production (Lopez Rodriguez et al., 2017).

Spermatogenesis is influenced by numerous endogenous and exogenous factors, among which the most important are exposure to high ambient temperatures, photoperiod, nutrition, ejaculation frequency, and housing conditions (Flowers, 2015). Each of these factors may induce specific alterations, which often allows for the identification of a correlation between the type of sperm malformation observed and the underlying factor contributing to reduced fertility (Bonet et al., 1993). The relationship between different parameters varies depending on the breed and individual boar (Knecht et al., 2014). Heat stress influences the movement characteristics and metabolic profile of sperm, but boars can differ in how strongly they respond to elevated temperatures (Sui et al., 2022). The main consequence of heat stress on the testes is the destruction of germ cells through apoptosis, with spermatocytes, spermatids, and spermatozoa in the epididymis being the most sensitive to this effect (Shahat et al., 2020). Although the negative impact of elevated temperature on semen quality during in vitro exposure has been well documented, the specific molecular mechanisms triggered by heat in mature spermatozoa remain insufficiently understood and require further investigation (Li et al., 2023). Ultrastructural anomalies of boar sperm are divided into head and tail malformations. Observed changes include enlarged or vacuolated acrosomes, nuclear irregularities, swollen or excessive mitochondria, and various tail deformities such as coiling, folding, vesicle formation, and fusion of two tails (Bonet et al., 1993). A shorter flagellum and a higher head-to-flagellum length ratio are characteristic of spermatozoa from low volume ejaculates (Górski et al., 2021).

According to Savić (2014), there are also malformations such as spermatozoa with proximal and distal droplets, which are classified as secondary abnormalities based on their origin. Depending on their origin, sperm abnormalities are generally classified into two groups: primary malformations, which develop in the testes during spermatogenesis, and secondary malformations, which occur later, in the epididymis, during sperm maturation (Briz et al., 1996).

The aim of this study was to investigate the relationship between ejaculate traits and the occurrence of abnormal spermatozoa in boars exposed to summer heat stress.

Materials and Methods

This paper represents a continuation of the research Petrović et al. (2024), and therefore the methodology remains the same. The study involved seventeen boars from two breeds: four Landrace and thirteen Large White. Boars aged 2 to 3 years. The analysis included 129 ejaculates in total; however, the sample size differed across traits. The evaluation of boar fertility was conducted during the August - October period, which is regarded as the most challenging time of the reproductive season. The analysis included several key indicators of semen quality, including ejaculate volume (VOL, ml), sperm concentration (CON, $\times 10^6$/ml), and total spermatozoa count (NT, $\times 10^9$; calculated as VOL × CON). Functional spermatozoa count (NF, $\times 10^9$) was estimated by multiplying NT with the percentage of motility in native semen (MOTN, %). Additionally, motility after dilution (MOTD, %), number of insemination doses produced (NPD), and sperm viability expressed as percentages of live (PL, %) and dead (PD, %) spermatozoa were assessed. Morphological evaluation included the proportion of spermatozoa with protoplasmic droplets (PPK, %), as well as abnormalities of the tail (PPLJR, %), head (POTKG, %), and acrosomal region (PAKR, %).

VOL was measured using a graduated cylinder and expressed in milliliters (±2 ml accuracy). Sperm concentration in native semen was determined by photo-colorimetry using a Magacell device (Magapor, Spain). Semen samples were diluted with a commercial extender (Vitasem, Magapor, Spain). MOTN and MOTD were assessed subjectively under a light microscope (BA410, Motic®, USA) using a ×100 objective. NT was calculated as the product of ejaculate volume and sperm concentration, while the NF was obtained by multiplying NT by MOTN. Sperm viability was evaluated by eosin-nigrosin staining, following the protocol described by Savić and Petrović (2019). Using the applied staining technique, viable spermatozoa appeared unstained against a dark background, while non-viable cells were partially or completely stained. The evaluation of pathological sperm forms was conducted microscopically on the same permanent slide using an appropriate immersion objective. A total of 100 spermatozoa were examined, and the number of spermatozoa exhibiting a particular abnormality was expressed as a percentage of the total count. Prepared slides were examined under the same microscope (BA410, Motic®, USA) at ×400 magnification. Morphological abnormalities were evaluated on permanent stained slides using an immersion objective. For each sample, 100 spermatozoa were examined, and the

frequency of specific abnormalities (protoplasmic droplets, head, tail, and acrosomal defects) was expressed as a percentage of the total count.

Statistical analysis was performed using the SAS software package, version 9.3 (SAS Institute Inc., 2002–2010). Relationships between the examined traits were evaluated using Pearson's correlation coefficient. The strength of the correlations was interpreted based on the classification proposed by Petz (2004), as follows:

0.00-0.20 - negligible,
0.20-0.40 - weak,
0.40-0.70 - moderate, and
0.70-1.00 - strong correlation.

Results and Discussion

Descriptive analysis of boar semen traits revealed considerable individual variation. The average ejaculate volume was approximately 292 ml, while sperm concentration reached around 367 $\times 10^6$/ml. The total and functional sperm counts were 107.5 $\times 10^9$ and 90.8 $\times 10^9$, respectively. Native motility averaged 84%, with post dilution motility slightly lower at 78%. On average, 15 insemination doses were obtained per ejaculate. Regarding sperm viability from native ejaculates, live spermatozoa made up approximately 76% of the sample, while 24% were non-viable. The most frequent morphological defect was the presence of a protoplasmic droplet (13.6%), followed by tail (2.5%), head (1.3%), and acrosomal (0.2%) abnormalities, all showing high variability.

A correlation of varying strength, direction, and statistical significance was established between the examined semen traits (Table 1). A strong positive correlation ($r_f = 0.770$; $p<0.001$) was found between ejaculate volume and both the total sperm count and the number of functional spermatozoa. Similar findings were reported by Smital et al. (2005). A positive, but moderate, correlation ($r_f = 0.423$; $p<0.001$) was also observed between ejaculate volume and the number of insemination doses produced per ejaculate. These results suggest that ejaculates with larger volumes contain higher total and functional sperm counts, enabling the production of a greater number of insemination doses. This is consistent with the findings of Knecht et al. (2014) and Górski et al. (2017).

There is a weak negative correlation between ejaculate volume and sperm motility, which is statistically significant ($p<0.05$) only in the case of the motility percentage of native semen. Górski et al. (2017) also reported a weak negative relationship between these traits, although it was not statistically significant in their study. According to Savić (2014), a higher ejaculate volume does not necessarily imply greater fertility, as ejaculates with larger volumes may contain a lower proportion of progressively motile or functionally competent spermatozoa.

Table 1. Correlations between the examined semen traits

Trait	Parameter	CON	NT	NF	MOTN	MOTD	NPD	PD	PL
VOL	r_f	-0.132	0.770	0.714	-0.190	-0.186	0.423	0.169	-0.169
	p	0.188	0.000	0.000	0.037	0.052	0.000	0.122	0.122
CON	r_f		0.492	0.537	0.247	0.282	0.183	-0.269	0.269
	p		0.000	0.000	0.013	0.056	0.075	0.017	0.017
NT	r_f			0.983	-0.088	-0.098	0.519	0.002	-0.002
	p			0.000	0.383	0.344	0.000	0.988	0.988
NF	r_f				0.085	0.021	0.508	-0.030	0.030
	p				0.401	0.839	0.000	0.794	0.794
MOTN	r_f					0.685	-0.085	-0.242	0.242
	p					0.000	0.374	0.028	0.028
MOTD	r_f						-0.098	-0.454	0.454
	p						0.310	0.000	0.000
NPD	r_f							0.014	-0.014
	p							0.897	0.897
PD	r_f								-1.000
	p								0.000

VOL - ejaculate volume, CON - sperm concentration, NT - total spermatozoa count, NF - functional spermatozoa count, MOTN - native sperm motility, MOTD - sperm motility after dilution, NPD - number of produced doses, PD - percentage of dead spermatozoa, PL - percentage of live spermatozoa.

On the other hand, a weak positive correlation ($r_f = 0.247$; $p = 0.013$) was observed between sperm concentration and the motility of native semen. These coefficients suggest that more concentrated ejaculates exhibited better mass motility. However, it should be noted that sperm motility was subjectively evaluated.

A weak positive correlation ($r_f = 0.269$; $p<0.05$) was also found between sperm concentration and the proportion of live spermatozoa, while a weak negative correlation ($r_f=0.269$; $p<0.05$) was found with the proportion of dead spermatozoa, indicating that ejaculates with higher concentration had a greater number of viable sperm. Furthermore, sperm concentration showed a moderate positive correlation ($r_f>0.400$; $p<0.001$) with both the total number and number of functional spermatozoa. However, it should be emphasized that these two traits (total number – NT and functional number – NF) are calculated values. Therefore, ejaculates with higher sperm concentrations were found to contain a greater total and functional sperm count. In contrast, Knecht et al. (2014) reported that the correlation between concentration and total sperm count ranged from negligible to weak, depending on the breed. A strong positive correlation ($r_f = 0.983$; $p<0.001$) was established between the total and functional sperm count, which is expected, as NF is derived

from NT. Moderate correlations (r_f = 0.519; r_f = 0.508; p<0.001) also suggest that ejaculates with a higher total and functional sperm count yielded a greater number of insemination doses. Similar results were reported by Knecht et al. (2014), who found an almost perfect correlation ($r_f \approx 1$; p<0.01) between total sperm count and the number of doses produced. A moderately strong positive correlation (r_f = 0.685; p<0.001) was observed between the motility of native semen and motility after dilution. Weak correlations between native sperm motility and the percentages of dead and live sperm indicate that ejaculates with better motility in the native state tend to contain a lower proportion of dead and a higher proportion of live sperm. Additionally, a moderate correlation (r_f = 0.454; p<0.001) between motility after dilution and the proportion of live sperm implies that better post-dilution motility is associated with greater sperm viability.

Correlations between various types of sperm abnormalities are presented in Table 2. Weak positive correlations (r_f > 0.200) of varying statistical significance (p<0.05; p<0.01) were found, particularly between secondary abnormalities (excessive proximal cytoplasmic droplets) and primary abnormalities involving the tail and acrosome. Weak positive correlations (r_f > 0.200) of varying statistical significance (p<0.05; p<0.01) were found, particularly between secondary abnormalities (excessive proximal cytoplasmic droplets) and primary abnormalities involving the tail and acrosome. Weak correlations were also observed among different primary anomalies. These results contradict the findings of Kamanova et al. (2017), who reported a strong negative correlation between spermatozoa with proximal cytoplasmic droplets and those with acrosomal defects. Moreover, the authors concluded that there were moderate to strong negative correlations between primary head and tail abnormalities, with the exception of bent-tail spermatozoa, which were strongly positively correlated with head defects.

Table 2. Correlation between pathological forms of spermatozoa

Trait	Parameter	PPLJR	POTKG	PAKR
PPK	r_f	0.270	0.165	0.213
	p	0.005	0.090	0.028
PPLJR	r_f		0.273	0.060
	p		0.004	0.536
POTKG	r_f			0.226
	p			0.019

PPK - percentage of spermatozoa with a protoplasmic drop, PPLJR - percentage of spermatozoa with a pathological shape on the tail, POTKG - percentage of spermatozoa with pathological changes on the head, PAKR - percentage of spermatozoa with pathological changes on the acrosomal part

Among the semen traits, only the motility of native semen and semen after dilution, as well as the number of insemination doses produced, were found to be correlated with pathological forms of spermatozoa (Table 3). Based on the obtained weak negative correlation coefficients, it can be concluded that ejaculates containing a lower proportion of spermatozoa with secondary (excess cytoplasmic droplets) and primary tail defects are expected to exhibit better motility and result in a higher number of insemination doses. These findings are consistent with the earlier study by Barna et al. (2021), who reported that ejaculates with better motility had a lower proportion of spermatozoa exhibiting cytoplasmic droplets and acrosomal damage.

Table 3. Correlation between semen traits and pathological forms of spermatozoa

Trait	Parameter	PPK	PPLJR	POTKG	PAKR
VOL	r_f	0.103	0.026	-0.133	-0.078
	p	0.349	0.815	0.225	0.477
CON	r_f	-0.040	-0.169	-0.103	-0.170
	p	0.725	0.136	0.365	0.134
NT	r_f	-0.106	-0.094	-0.152	-0.188
	p	0.988	0.408	0.181	0.096
NF	r_f	-0.145	-0.145	-0.185	-0.172
	p	0.206	0.205	0.104	0.132
MOTN	r_f	-0.232	-0.319	-0.151	-0.078
	p	0.035	0.003	0.173	0.487
MOTD	r_f	-0.239	-0.220	-0.007	-0.023
	p	0.030	0.047	0.952	0.840
NPD	r_f	-0.243	-0.113	-0.132	-0.024
	p	0.025	0.304	0.231	0.826
PD	r_f	0.069	0.104	0.133	0.086
	p	0.478	0.287	0.171	0.376
PL	r_f	-0.069	-0.104	0.133	-0.086
	p	0.478	0.287	0.171	0.376

VOL - ejaculate volume, CON - sperm concentration, NT - total spermatozoa count, NF - functional spermatozoa count, MOTN - native sperm motility, MOTD - sperm motility after dilution, NPD - number of produced doses, PD - percentage of dead spermatozoa, PL - percentage of live spermatozoa, PPK - percentage of spermatozoa with a protoplasmic drop, PPLJR - percentage of spermatozoa with a pathological shape on the tail, POTKG - percentage of spermatozoa with pathological changes on the head, PAKR - percentage of spermatozoa with pathological changes on the acrosomal part

Ejaculate volume was not correlated with pathological forms of spermatozoa, which contrasts with the findings of Górski et al. (2021), who reported a slight dependence of anomaly frequency on ejaculate volume.

According to their study, spermatozoa from low-volume ejaculates had shorter tails and a higher head-to-tail length ratio compared to those from medium- and high-volume ejaculates. Kondracki et al. (2020) investigated the impact of sperm concentration on sperm morphology and concluded that in approximately 50% of the ejaculates, spermatozoa with proximal and distal cytoplasmic droplets were observed, and their frequency increased with rising sperm concentration. In contrast, no correlation was found in our study between sperm concentration and pathological sperm forms. Understanding the interaction between sperm morphology particularly cytoplasmic droplets and sperm quality is essential to avoid reduced fertilization efficiency (Henning et al., 2021).

Conclusion

The analysis of the examined semen traits showed correlations of different strength, direction, and significance. Ejaculate volume was strongly positively related to the total and functional sperm count, and moderately related to the number of insemination doses. This suggests that larger ejaculates with more sperm cells can produce a greater number of doses. On the other hand, there was a weak negative correlation between volume and sperm motility, which was statistically significant only for the motility of native semen. Sperm concentration had a moderate positive correlation with both the total and functional sperm count, and a weak positive link with the percentage of live sperm. Native and diluted semen motility were moderately correlated with each other, while their relationship with the percentage of live or dead sperm cells was weaker. When it comes to abnormal sperm forms, weak positive correlations were found between secondary defects and some primary defects, especially those affecting the tail and acrosome. Interestingly, only native and diluted semen motility, along with the number of produced doses, showed a negative correlation with these abnormalities. This means that ejaculates with fewer defective sperm cells are likely to have better motility and yield more doses, making them more useful for artificial insemination.

Korelacija između osobina ejakulata i pojave patoloških oblika spermatozoida kod nerasta tokom perioda letnjeg toplotnog stresa

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Rezime

Sezonski toplotni stres poznat je kao faktor koji negativno utiče na plodnost nerasta, pre svega kroz smanjenje kvaliteta semena i povećanje učestalosti patoloških oblika spermatozoida. Cilj ovog istraživanja bio je da se ispita povezanost između osobina ejakulata i morfoloških anomalija spermatozoida kod nerasta tokom najosetljivijeg perioda u godini. U periodu od avgusta do oktobra prikupljeno je ukupno 129 ejakulata od 17 nerasta, u uslovima povišene spoljašnje temperature. Analizirani su zapremina ejakulata, koncentracija, ukupan i funkcionalni broj spermatozoida, pokretljivost (u nativnom i razređenom semenu), vitalnost, kao i prisustvo morfoloških anomalija - defekata glave, repa, akrozoma i protoplazmatskih kapljica. Rezultati su pokazali da su samo pokretljivost semena i broj proizvedenih doza bili u značajnoj korelaciji sa pojavom patoloških oblika spermatozoida. Utvrđena je slaba, ali dosledna pozitivna korelacija (r_f> 0,200; $p<0,05$ ili $p<0,01$) između određenih kategorija anomalija, naročito između sekundarnih oblika (protoplazmatska kapljica) i primarnih defekata na repu i akrosomalnoj regiji. Među primarnim anomalijama zabeležene su uglavnom slabe povezanosti. Ovi rezultati ukazuju na značaj praćenja pokretljivosti i morfologije spermatozoida kao ranih pokazatelja toplotnog stresa kod nerasta.

Ključne reči: nerast, kvalitet semena, patološke forme spermatozoida, osobine ejakulata, toplotni stress

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RELATIONSHIP BETWEEN BODY WEIGHT, BODY MEASUREMENTS AND CARCASS CHARACTERISTICS OF NILE TILAPIA (*Oreochromis niloticus* L.)

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Abstract: This study was conducted to evaluate the relationship between the body weight (BW), body measurements and carcass characteristics to determine the usefulness of body measurements in prediction of BW and fillet and carcass weight of Nile tilapia (*Oreochromis niloticus* L.). In the study, a total of thirty-six adult male tilapia specimens were used. The fish were killed and weighed individually using an electronic scale. Six linear body measurements were recorded: total length (TOL), standard length (STL), body width (BWI), body perimeter (BPE), body height (BHE) and head length (HEL). Likewise, the fish were processed to determine the carcass weight (CWE), viscera weight (VIW), head weight (HEW), fillet weight (FIW), backbone weight (BAW) as well as carcass (CYI) and fillet (FIY) yields. The results of the study indicated that the mean values of BW, body measurements and carcass characteristics presented low to moderate variability, varying from 3.45% for TOL to 26.74% for HEW. The Pearson correlation coefficients ranged from moderate to high ($0.342 \leq r \leq 0.869$). All the linear body measurements, except HEL, showed positive correlations with BW ($P<0.001$). Also, BW showed positive correlations ($P<0.05$; $P<0.001$) with the CWE ($r= 0.869$), FIW ($r= 0.638$), VIW ($r= 0.395$) and BAW ($r= 0.360$). The results of the stepwise regression analysis revealed that BW can be moderately predicted using BHE and STL as the predictor's variable. Besides, BW was the traits of higher importance in the prediction of CWE ($R^2 = 75.6\%$) and FIW ($R^2 = 40.7\%$). These findings contribute to decision making related to breeding programs of this aquaculture species.

Key words: aquaculture, body weight, fillet yield, *Oreochromis niloticus*, prediction equations.

Introduction

Globally, the aquaculture sector plays a relevant role in the food industry, since it contributes to meet growing food demand and address nutritional deficiencies with lower environmental impact than others animal source products. In recent decades, world aquaculture has increased the availability of aquatic foods, growing from 13.1 million mt in 1990 to 82.1 million mt in 2018 (Garlock et al., 2022).

In Mexico, the growth rate of production and the value of aquaculture production for the period 2008-2018 registered a growth of 9.1%, going from 1.7 to 2.1 million mt, respectively (SADER, 2018). The increase in this volume has been with species such as shrimp, catfish, tilapia, trout, red snapper, among others. It should be noted that shrimp and tilapia are the species that especially drive the positive performance of this sector. According to CONAFAB (2021), 65,000 tons are produced in the country through controlled aquaculture; however, it is not enough to cover the demand since 75,000 tons are imported, placing Mexico as the second largest importer of tilapia in the world, after the United States.

Currently, Nile tilapia (*Oreochromis niloticus* L.) is a popular species in both aquaculture as well as capture fisheries, and is considered a nutritious and relatively inexpensive alternative for human consumption (Aguilar-Moreno and Aguilar-Aguilar, 2024). This aquaculture species has important characteristics such as rapid growth, resistance to disease, high adaptability to different environments, food, as well as water quality. In Mexico, the systems used for its breeding range from the most rudimentary (extensive) to technologically advanced farms (intensive) (Vega-Villasante et al., 2010). Its cultivation is more frequent in tropical areas, where concrete or other material ponds are established to carry out the sowing, pre-fattening and fattening of tilapia, usually near natural bodies of water such as rivers, lagoons and lakes (Aguilar-Moreno and Aguilar-Aguilar, 2024).

On the other hand, through genetic improvement of livestock species seeks the increase of economically important traits, such as the body weight (BW). This involves the selection and reproduction of the individuals that present the best performance for the desirable trait, which can be directly or indirectly measured by means of others that are correlated with it, as body measurements (LBM) (Oliveira et al., 2019). Due to the preference and popularity of tilapia production among farmers, comprehensive studies must be conducted on the relationship between the BW, LBM and carcass characteristics. Understanding these characteristics and their potential correlation will contribute to broader knowledge of tilapia breeding and management strategies (Allaman et al., 2013). Body shape, weight and

relationship between body measurements has been studied because the relation not only fillet weight but also fillet and carcass yield (dos Santos et al., 2019).

Therefore, this study was conducted to evaluate the relationship between the body weight (BW), body measurements and carcass characteristics to determine the usefulness of body measurements in prediction of BW and fillet and carcass weight of Nile tilapia (*Oreochromis niloticus* L.).

Materials and Methods

Location and biological material

A total of thirty-six adult male tilapia with an age between 160 and 180 days were captured manually in different ponds of an aquaculture farm located in the community of Bécal, Calkiní, Campeche, México (20°24'55.1"N and 90°02'05.1" W at an altitude of 50 masl) and transferred to the Food Technology Laboratory of the Higher Technological Institute of Calkini for measurement and processing.

The study area presents a warm subhumid climate with summer rain (Aw). The average annual temperature and total annual precipitation ranged from 22.1 to 28.1°C and 853.3 to 1756.9 mm, respectively (INEGI, 2021). The animals studied were under the same culture conditions. Stocking density varied according to the growth stage of fish (100-120 fish/m^{-3} at the stocking stage and 20-50 fish/m^{-3} at the fattening stage). The ponds are made of geomembrane (6.0 m diameter × 1.2 m high) and are supplied with deep well water through a recirculation system, which is maintained at a neutral pH and an average temperature of 28.5ºC. Fish were fed three times a day with a daily ration of commercial feed (ADM® GROWFISH) corresponding to 1.5-2% of total body weight at the growing stage. Subsequently, it was gradually reduced to reach 1% of total body weight.

Morphometric measurements

The animals were killed by stunning and weighed individually using a digital analytical balance (Medidata®) with a precision of 0.01 g. All fish were processed according to the Official Mexican Standards (NOM-008-ZOO-1994, NOM-009-ZOO-1994, and NOM-033-ZOO-1995) established for the humane slaughter of animals intended for meat production.

Six linear body measurements were recorded following the methodology described by Fernandes et al. (2015) and dos Santos et al. (2019); these were: total length (TOL), standard length (STL), body width (BWI), body perimeter (BPE), body height (BHE) and head length (HEL) (Figure 1). The body measurements were measured using a tape measure graduated in cm and a digital calliper (Mitutoyo®) with an accuracy of 0.01 mm. To ensure minimal errors the measurements were done by a single operator.

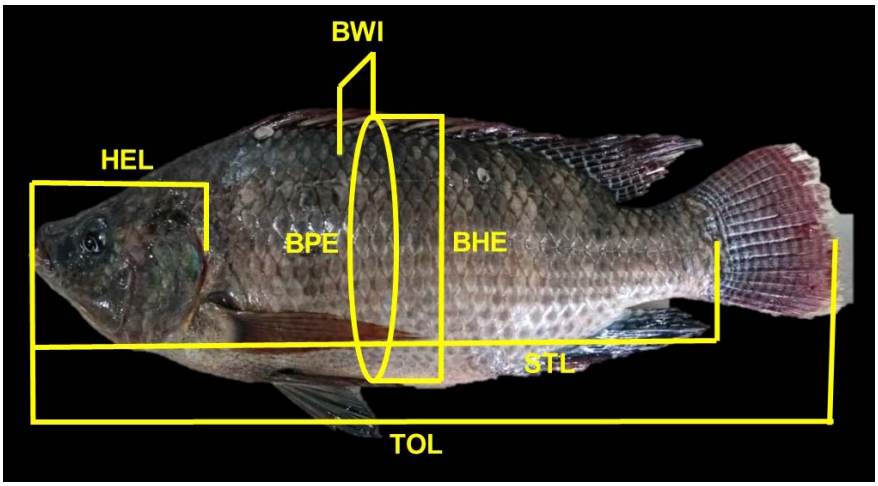

Figure 1. Schematic representation of the morphometric measurements taken in Nile tilapia (*Oreochromis niloticus* L.). TOL: Total length, STL: Standard length, BWI: Body width, BPE: Body perimeter, BHE: Body height, HEL: Head length.

Slaughter and carcass characteristics

The processing of the animals was carried out following the methodology proposed by Melo et al. (2013). Briefly, the fish were eviscerated to obtain the carcass weight (CWE) which consists in total weight of carcass with head minus the viscera. The latter were weighed separately to obtain the viscera weight (VIW). After, the skin was removed along with the flakes in the direction 'skull to tail'. The head was separated from the trunk by cutting in the line after the caudal end of the operculum and weighed to determine the head weight (HEW). With a filleting knife, the fillets were removed, without the two lateral ribs of the fish that are present longitudinally along the entire length of the spine and ribs and weighed to determine the total fillet weight (FIW). The backbone weight (BAW) without meat was also determined individually.

Carcass (CYI) and fillet (FIY) yields were determined as the percentage (%) of BW (whole fish).

Data analysis

Normal distribution of the variables was analysed according to the Shapiro-Wilk test. All data were analysed using descriptive statistics using the SAS software, version 9.4 (SAS Institute Inc., Cary, NC). Pearson correlation coefficients (r) were estimated using the PROC CORR procedure to evaluated relationship between BW, linear body measurements and carcass characteristics. The Stepwise regression analysis was used to obtain equations for predicting BW,

CWE and FIW from BW and linear body measurements so that only the significant (P<0.05) predictor variables were included in the model.

Results and Discussion

Descriptive statistics of BW, linear body measurements and carcass characteristics of Nile tilapia are presented in Table 1. The mean BW was 523.35 ± 52.90, while mean linear body measurements were 31.29 ± 1.08 cm, 25.91 ± 0.96 cm, 4.38 ± 0.28 cm, 23.77 ± 0.87 cm, 10.07 ± 0.38 cm and 7.95 ± 0.40 cm for TOL, STL, BWI, BPE, BHE and HEL, respectively. In general, mean values of BW, linear body measurements and carcass characteristics exhibited moderate to high variability, ranged from 3.66% for BPE to 26.74% for HEW. According to Sam et al. (2020), the moderate variability in some traits as carcass characteristics in domestic animals indicate that these traits could respond to selection and should be exploited.

The results of BW and linear body measurements observed in this study were lower to those reported by Oliveira et al. (2019) for of tilapia male breeders. However, were higher compared to those reported by Melo et al. (2013) for tilapia with BW ranged to 400 to 599 g. The differences between the tilapia groups studied may be due to either genetic differences or environmental factors (Kosai et al., 2014). In addition, it is known that in fish farming variations in size among animals are generally related to competition for food within a population and the resulting feeding hierarchy (dos Santos et al., 2019).

Table 1. Descriptive statistics of body weight, body measurements and carcass characteristics of Nile tilapia (*Oreochromis niloticus* L.)

Traits	Mean	Standard deviation	Minimum	Maximum	CV (%)
BW (g)	523.35	52.90	418.00	657.00	10.10
TOL (cm)	31.29	1.08	29.40	34.20	3.45
STL (cm)	25.91	0.96	24.30	28.00	3.70
BWI (cm)	4.38	0.28	3.80	4.90	6.39
BPE (cm)	23.77	0.87	22.40	26.90	3.66
BHE (cm)	10.07	0.38	9.10	10.90	3.77
HEL (cm)	7.95	0.40	7.20	8.70	5.03
FIW (g)	197.25	24.75	148.00	263.70	12.54
VIW (g)	56.85	7.94	42.10	79.30	13.96
BAW (g)	149.97	35.12	91.30	226.70	23.41
HEW (g)	102.61	27.44	73.00	182.10	26.74
CWE (g)	363.88	46.75	286.60	513.80	12.84
CYI (%)	69.46	4.28	60.62	79.17	6.16
FIY (%)	37.75	3.52	29.43	46.40	9.32

CV: Coefficient of variation, BW: Body weight, TOL: Total length, STL: Standard length, BWI: Body width, BPE: Body perimeter, BHE: Body height, HEL: Head length, FIW: Fillet weight, VIW:

Viscera weight, BAW: Backbone weight, HEW: Head weight, CWE: Carcass weight, CYI: Carcass yield, FIY: Fillet yield.

The phenotypic correlation matrix between BW, linear body measurements and carcass characteristics of Nile tilapia is represented in Figure 2. In general, the correlation coefficient varied from moderate to high ($0.342 \leq r \leq 0.869$). All the body measurements, except HEL, showed positive correlations ($P<0.001$) with BW. This indicates that selection for any of these body measurements, in the order > BHE > STL > BPE > TOL > BWI, will lead to improvement in BW of Nile tilapia. Highest correlation were recorded between BW and BHE ($r = 0.605$). Similarly, it is an indication that this body dimension could serve as a predictor of BW under field conditions (Ogah, 2011). In addition, the results in the present study also showed that interrelationships exist between linear body measurements as correlation coefficients were positive and significant.

Similar finding were reported by da Silva et al. (2018) in Nile tilapia, GIFT-Epagri strain, where body weight showed highest correlations with total length ($r = 0.968$ and 0.956) and standard length ($r = 0.957$ and 0.950) for both males and females, respectively. According to Kosai et al. (2014), the length-weight relationship of fish is important in aquaculture, because it allows the estimation of average BW of the animals of a given length group by establishing a mathematical relation between the two. Also, is often used as an parameter of fatness, general well-being and regional comparison.

For its part, Hassanien et al. (2011) they reported a low and positive correlation between body thickness and body depth ($r = 0.433$), a high and positive correlation between body length and tail length ($r= 1.00$), as well as a high and negative correlation between head length and trunk length ($r = -1.00$) in cultured Nile tilapia.

Regarding the carcass characteristics, BW showed positive and significant ($P<0.05$; $P<0.001$) correlations only with the CWE ($r = 0.869$), FIW ($r = 0.638$), VIW ($r = 0.395$) and BAW ($r = 0.360$). The positive correlations recorded in this study for some carcass characteristics and BW indicate that these characteristics are influenced by the same genes in the same direction; that is to say, the traits may be under the same genetic influences (Okpeku et al., 2011). Thus, selection and improvement for BW will lead to improvement of carcass characteristics (Sam et al., 2020). Specifically, BWI indicated higher correlation with the majority of the carcass characteristics estudied, except that it negatively correlated with FIY ($r = -0.584$) and HEW ($r = -0.376$).

Meanwhile, FIW showed positive correlations ($P<0.001$) with BPE ($r = 0.612$), TOL ($r = 0.464$), STL ($r = 0.390$) and BHE ($r = 0.354$). The importance of slaughter traits, as fillet weight and yield, for the fish industry is well known (Fernandes et al., 2015). The fillet is the presentation of the final product that is most marketed by the fish processing industry, especially when compared to the

gutted whole fish (Melo et al. 2013). In the study of Melo et al. (2013), the FIY of of Nile tilapia from different BW class had the highest correlation coefficients with the ratio body width/head length. In that study, other variables were important, such as the body height.

In another study by Oliveira et al. (2019), some characteristics of tilapia breeders presented a moderate correlation, such as between FIY and FIW (r = 0.55) in females and others presented a low correlation, such as between BW and ultrasound area (r = 0.03) in males. The authors also observed strongly correlated characteristics such as average daily gain and BW (r = 0.99) in females.

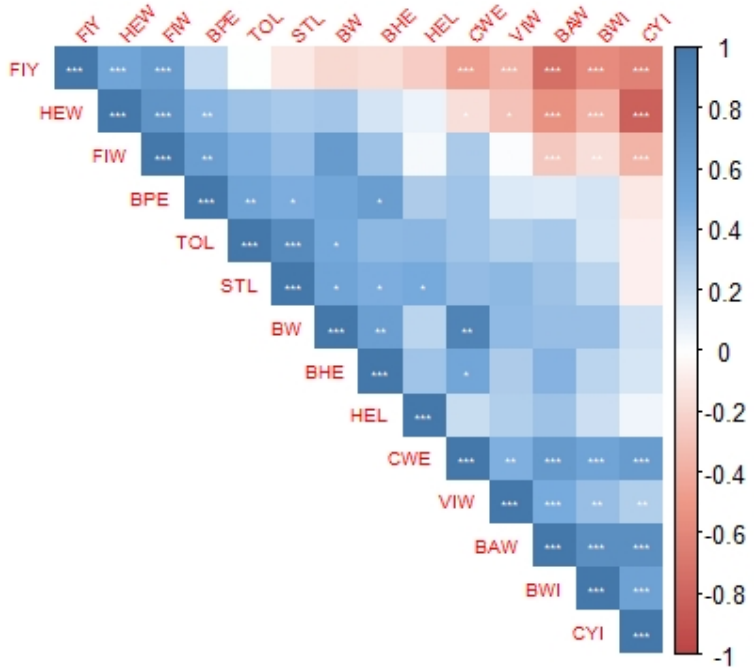

Figure 2. Heat map of Pearson's correlations (r) between body weight, body measurements and carcass characteristics of Nile tilapia. Blue color means high correlation (P<0.001), white color means mid-correlation (P<0.05), and red color means low correlation with negative direction.

Regression equations for predicting BW and carcass characteristics from body measurements of Nile tilapia using Stepwise regression analysis are presented in Table 2. The results showed that BW can be moderately predicted using BHE as the predictor variable (R^2 = 36.6%) (Eq. 1); however, predictive accuracy was improved slightly by adding the STL to the equation, since the value of the determination coefficient increased to 46.6% (Eq. 2). These results confirm the

importance of standard length in identifying tilapia with better carcass yield, which depends on body weight (Melo et al. 2013).

For CWE, the results of the stepwise analysis revealed that BW was the variable of higher importance in the prediction of CWE in Nile tilapia, where this trait alone accounted for 75.6% of the total variation in BW (Eq. 3). However, the combination with BWI increased the proportion of the explained variance to 83.0% (Eq. 4). These findings confirm the fact that carcass traits in domestic animals can be predicted from BW with accuracy. This probably might be as a result of the positive and correlated relationships of carcass traits with BW used in predictions.

For the prediction of FIW, three equations were obtained, however, the best equation (Eq. 7) explained 67.3% of the observed variation and considered BW, BWI and BPE as predictors. Thus, selection for BW is the most appropriate alternative for gains in FIY and FIW as described by da Silva et al. (2018).

With regard to variance inflation factor (VIF) values obtained in this study, it can be inferred the existence of moderate collinearity, since VIF values greater than 10.00 indicate severe collinearity rendering the reliability of the predictive equation not effective (Ogah, 2011).

Table 2. Prediction equations for body weight and carcass traits from linear body measurements of Nile tilapia.

Eq. No.	Equations	R^2	MSE	VIF
Body weight (BW, g)				
1	BW (g)= -305.39 + 82.22×BHE	0.366	1831.29	1.000
2	BW (g)= -583.27 + 59.50×BHE + 19.55×STL	0.466	1591.49	1.278
Carcass weight (CWE, g)				
3	CWE (g)= -38.12 + 0.76×BW	0.756	550.64	1.000
4	CWE (g)= -197.39 + 0.67×BW + 47.61×BWI	0.830	395.98	1.155
Fillet weight (FIW, g)				
5	FIW (g)= 40.95 + 0.29×BW	0.407	374.37	1.000
6	FIW (g)= 171.56 + 0.37×BW + -39.04×BWI	0.585	270.51	1.155
7	FIW (g)= -22.91 + 0.28×BW + -37.59×BWI + 9.93×BPE	0.673	220.31	1.414

BW: Body weight, TOL: Total length, STL: Standard length, BWI: Body width, BPE: Body perimeter, BHE: Body height, HEL: Head length, FIW: Fillet weight, CWE: Carcass weight.

Conclusion

In conclusion, it is possible to predict body weight of Nile tilapia with moderate accuracy from some linear body measurements as the body height and standard length. Additionally, the positive and significant relationship observed between these variables suggests that an increase in any of these body measurements leads to an increase in body weight. Likewise, the body weight is the most important trait for determining carcass characteristics of Nile tilapia. Therefore, these findings contribute to decision making related to improving breeding programs of this aquaculture species.

Veza između telesne mase, telesnih mera i karakteristika trupa nilske tilapije (*Oreochromis niloticus* L.)

Rodrigo Portillo-Salgado, Alondra Y. Sánchez-Márquez, Julio E. Oney-Montalvo, Dany A. Dzib Cauich, Emmanuel de Jesús Chi-Gutiérrez

Rezime

Ovo istraživanje je sprovedeno radi procene odnosa između telesne mase (BW), telesnih mera i karakteristika trupa, kako bi se utvrdila mogućnost korišćenja telesnih mera za predviđanje BW i težine fileta i trupa nilske tilapije (*Oreochromis niloticus* L.). U istraživanju je korišćeno ukupno trideset šest odraslih mužjaka tilapije. Ribe su žrtvovane i pojedinačno merene pomoću elektronske vage. Evidentirano je šest linearnih telesnih mera: ukupna dužina (TOL), standardna dužina (STL), širina tela (BWI), obim tela (BPE), visina tela (BHE) i dužina glave (HEL). Takođe, ribe su obrađene da bi se odredila težina trupa (CWE), težina unutrašnjih organa (VIW), težina glave (HEW), težina fileta (FIW), težina kičme (BAW), kao i prinos trupa (CYI) i fileta (FIY). Rezultati su pokazali da srednje vrednosti za BW, telesne mere i karakteristike trupa pokazuju nisku do umerenu varijabilnost, varirajući od 3,45% za TOL do 26,74% za HEW. Pirsonovi koeficijenti korelacije kretali su se od umerenog do visokog ($0.342 \leq r \leq 0.869$). Sve linearne telesne mere, osim HEL, pokazale su pozitivne korelacije sa BW ($P<0,001$). Takođe, BW je pokazala pozitivne korelacije ($P<0,05$; $P<0,001$) sa CWE ($r= 0,869$), FIW ($r= 0,638$), VIW ($r= 0,395$) i BAW ($r= 0,360$). Rezultati postupne regresione analize pokazali su da se BW može umereno predvideti korišćenjem BHE i STL kao prediktorskih varijabli. Pored toga, BW je bila osobina od većeg značaja u predviđanju CWE ($R2 = 75,6\%$) i FIW ($R2 = 40,7\%$). Ovi rezultati doprinose donošenju odluka u vezi sa programima uzgoja ove vrste akvakulture.

Ključne reči: akvakultura, telesna masa, prinos fileta, *Oreochromis niloticus*, jednačine predviđanja.

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

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NUTRIENT DIGESTIBILITY, GROWTH PERFORMANCE, CEACAL MICROBIAL PROFILE AND FERMENTATION CHARACTERISTICS OF RABBIT BUCKS FED *PILIOSTIGMA THONNINGII* ESSENTIAL OIL SUPPLEMENTED DIET

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Abstract: The purpose of this current experimental research (study) was to examine the effects of *Piliostigma thonningii* essential oil on the nutrient digestibility, growth characteristics, caecal microbes and fermentation characteristics of rabbits. The rabbits were randomly assigned to individual groups of 3, comprising precisely 15 rabbits per group, and were balanced for body weight so that each group's average initial body weight (BW) was similar—262.89 ± 22.36 g—in a fully randomised design. The first treatment (T1) was a basal control diet. In treatments 2 and 3, the basal control diet was supplemented with 2 ml PEO/kg diet and 4 ml PEO/kg diet respectively. Results for final body weight, total weight, average daily gains, feed conversion ratio, protein efficiency ratio, caecal *Lactobabacillus* spp, total volatile fatty acid ($p < 0.05$) improved with increasing level of PEO supplementation. Except for ether extract digestibility and acid detergent fibre digestibility, apparent digestibility of dry matter and all nutrients, caecal butyric and propionic acid were higher ($p < 0.05$) in T2 and T3 than in T1. Acid detergent fibre digestibility was higher ($P < 0.05$) in T3 than T1 with T2 being intermediate ($p > 0.05$) between T1 and T3, while the ether extract digestibility was highest in T3 ($p < 0.05$) and similar for T1 and T2 ($p > 0.05$). Caecal *E. coli*, Coliform bacteria and *Streptococcus* spp. counts and acetic acid were significantly higher ($p < 0.05$) in T1 than in T2 and T3. Caecal pH was not affected ($p > 0.05$) by treatments. Ammonia nitrogen was higher ($p < 0.05$) in T1 relative to T3 while T2 was intermediate between T1 and T3 ($p > 0.05$). It is concluded that the third group administered 4 ml PEO/kg diet is the optimum supplementation level, as it was more efficient in improving the performance of the

rabbit bucks and that *P. thonningii* essential oil supplementation improved feed utilisation, nutrient digestibility, growth performance of the experimental rabbits and reduced caecal pathogenic bacterial counts thereby improving health status of the experimental research bucks.

Key words: feed effects, feed utilization, gut bacteria and metabolism, phytogenic feed substance, pseudo ruminants.

Introduction

Livestock production in the tropics have faced several challenges in terms of poor performance and feed utilization coupled with the incessant misuse of synthetic antibiotics to boost production. These all have led to production of unhealthy and unwholesome meat for human consumption. The use of phytogenic feed additives has therefore been encouraged and currently been adopted to bring an end to this undesirable outcome in the finished products (Anaso and Alagbe, 2025). The aromatic qualities of plant materials where essential oils can be extracted and isolated are how Anaso (2023a) categorized essential oils. The primary source of essential oils, also known as volatile oils, is plant material. Specifically, fragrant oily liquids are extracted through distillation from plant components such flowers, buds, leaves, twigs, bark, and organic fruits and roots (Anaso, 2023a). Anaso (2023a) further described essential oils as steam-volatile or rather organic-solvent extracts that have been used traditionally for centuries in many parts of the world (Anaso et al., 2024a; Anaso et al., 2024b; Anaso et al., 2024c). They are mostly renowned for having a pleasant flavor and scent, as well as preservation characteristics. A wide variety of chemicals, including terpenes, alcohols, acetones, phenols, acids, aldehydes, and esters, are typically present in large quantities in essential oils (EOs) (Anaso et al., 2024b; Anaso, 2023b; Negi, 2012). These compounds can protect against bacterial, fungal, and insect attacks. EO studies have found numerous benefits in terms of feed utilisation, animal health, and rabbit live performance (Anaso, 2023a; Celia et al., 2016). Camel's Foot (*Piliostigma thonningii* Schum.), also known as Monkey Bread, is a small, crooked tree commonly found in savannahs. Belonging to the *Caesalpiniaceae* family and classified by Milne-Redhead, it has dark brown to black fissured bark (Anaso et al., 2024b). It has been discovered that Piliostigma thonningii possesses distinctive taste, antioxidant, insecticidal, and antibacterial properties. Nigerian traditionalists have long used it to treat dermatosis and malaria (Anaso et al 2024b; Anaso, 2023b). Although, a fair number phytogenics and EOs have been studied in livestock diets and had positive outcomes. Regarding the impact on rabbit growth traits, digestibility, and ceacal parametric values, little is known about camel's foot (Piliostigma thonningii Schum) EO as a continuation of research Anaso et al.

(2024b); Anaso et al. (2024c); Anaso (2023a); Anaso (2023b); Anaso et al., (2023).

Many essential oils and their plant extracts as reported by Simitzis (2017); Abd El-Azeem et al. (2019); Elghalid et al. (2020); El-nomeary et al. (2020) are highly acclaimed for their significant antioxidative effects, which are primarily determined by the specific phenolic compounds in the oil and other phytochemical fractions.

The goals of this investigation were, therefore, to:
1) Evaluate the nutrient digestibility of experimental rabbits
2) Access the growth parametric characteristics and
3) Determine the caecal microbial and fermentation characteristics of rabbits fed PEO supplemented diet.

Materials and Methods

Experimental site

The Monogastric Unit of the University of Abuja Teaching and Research Farm served as the site where the experiment was conducted. As a continuation to experiment carried out by Anaso et al. (2023). The project location is between longitudes 007020' and 007051' E and latitudes 08051' and 09037'N. The range of annual rainfall is 1,145–1,631 mm. In the rainy season, the temperature ranges from 25.8 to 30.2°C, whereas in the dry season, it is between 36 and 42°C. According to Anaso et al. (2023) and Itiowe et al. (2019), relative humidity is roughly 60% during the rainy season and 30% during the dry season.

Collection of P. thonningii seeds and extraction of the essential oil

The Piliostigma thonningii seeds were obtained from the southern Guinea savannah agro ecological zone using the same methods as Anaso et al. (2023). The seed was identified and confirmed at the Department of Biological Science by a qualified taxonomist from the Forestry Research Institute of Nigeria (FRIN) similar to researches conducted (Anaso et al., 2024b; Anaso et al., 2024c; Anaso and Alagbe, 2025).

The essential oil was extracted using the steam distillation process. Using the Clevenger apparatus, the essential oil was extracted in accordance with the methodology that Anaso et al. (2023) adopted from Mohamed et al. (2006). Before being extracted, the P. thonningii seeds were finely crushed, shade-dried, and kept at room temperature. About 100 g of dried ground sample was suspended in 700 ml of distilled water using a distillation process at 100°C for about three hours, as described by Anaso (2023b), where the condenser was connected to a water input and exit, and the ground seed sample was placed in a steel device to heat up and soften the essential oil in vaporized form (Anaso et al., 2024b; Anaso et al., 2024c).

The resultant vapourized essential oil droplets congregated in a cooling system after mixing with steam, the carrier. Using the same process as Anaso et al. (2024b); Anaso et al. (2024d); Anaso et al. (2023), the essential oil was then extracted using a collection tube, and the following formula was used to determine the percentage of oil content.

% Oil content (v/w) = volume of the oil extracted (ml)/weight of the sample taken (g) x 100

Ethical Acceptance

After the research proposal was presented on November 17, 2022, the Animal Ethics and Conduct Board of the Department of Animal Science University of Abuja, Nigeria, gave ethical approval with approval registration number 19/501/ANSJ/002. The approval, which was given prior to the thesis's advancement, was closely followed and examined to ensure that it complied with international guidelines for rabbit research (Kiani et al., 2022; Anaso and Olafadehan, 2025).

Management and treatment of experimental animals

The trial was a follow-up to an experiment by Anaso et al. (2023) in which 45 male Dutch rabbits, at 5 weeks of age, weighing approximately 262.89 ± 22.36 g and in good clinical health, were weaned. The rabbits were purchased from the National Animal Production Research Institute (NAPRI, ABU, Zaria) in Nigeria. The hutches and the area around them were completely cleaned, including sweeping and washing, two weeks before to the rabbits' arrival. After that, Hypo® (sodium hypochlorite, caustic soda, and de-mineralized water) and Morigad antiseptic were used to disinfect the area. The animals were kept apart and given preventative care for a period of two weeks. As part of the treatment, drinking water was infused with electrolyte supplement (Vitalyte®). To control endo and ecto parasites, an intramuscular parenteral injection of the broad-spectrum antibiotic oxytetracycline HCl at 1.0 mL/10 kg BW and a subcutaneous injection of an anti-parasitic medication (Avomec®) at 0.5 mg/kg of the animal body weight (BW) were used. In addition, at the start of the study, rabbits received a single 1mL subcutaneous injection of coccidiostat (Sulphadimidine Sodium BP solution), administered in accordance with the manufacturer's recommendations.

The rabbits were kept in separate metabolic hutches with open sides so that urine and faecal waste could be kept apart. As described in previous research by Anaso et al 2024b, a strong disinfectant was used every day to clean the hutches. A weighing scale was used to weigh each rabbit individually in order to determine their starting body weight. Three groups of fifteen rabbits each were formed. They were balanced for BW so that rabbits in each group had a comparable average

beginning BW and then assigned to one of three treatments in a completely randomised design.

A control diet was formulated in compliance with the National Research Council's (NRC's) 1994 guidelines for growing rabbits. For a duration of 12 weeks, water and food were given freely, with feeding occurring twice a day at approximately 8:00 a.m. and precisely 4 p.m. Rabbits in T1 (first group) were fed a basal/control (general) diet. In the other treatments, the control (general) diet was supplemented precisely two and four mL of Piliostigma thonningii essential oil per kilogram of the general diet.

Table 1. Composition of ingredients (%) in the experimental diet

Ingredient	Quantity
Corn	30.00
Bean husk	20.00
Soy flour	7.00
Maize bran	20.00
Peanut cake	19.40
Bone-based compost	2.00
Sodium chloride	0.30
Calcium carbonate	1.00
Vitamin: Mineral Premix	0.30
Total Quantity	100.00

Growth performance

Precise weight of individual experimental animal was determined weekly using a commercial weighing scale. Total weight gain (TWG) was obtained by subtracting the starting (initial) body weight (INBW) from the final body weight (FBW), while the average daily weight gain (ADWG) was obtained by division of the TWG by the complete number of experimental days, feed conversion ratio as the division of the average daily feed intake (ADFI) by the average daily gain, protein efficiency ratio (PER) as the division of the ADG by the crude protein intake all as explained and stated by Olafadehan et al. (2014) using the mathematical equations: TWG = FBW – INBW; ADG = TWG/Experimental days; FCR = AFI/ADG; PER = ADG/CPI.

Digestibility trial

At the 15th week of the experiment at 5 months of age of experimental rabbits, for the final 7 days of the trial, daily feacal samples were obtained in the early hours of the day prior to feeding to determine total tract digestibility. Every day, faecal sample was obtained from each treatment's animals using the technique

outlined by Aregheore (2004). The daily faecal production for each animal under the particular treatment was calculated, and twenty percent of the total amount collected was reserved for the analysis of dry matter. Samples of the excrement were oven dried for 48 hours at 70°C in a force-air oven, according to Olafadehan et al. (2014). The dried faecal samples of individual rabbit were then bulked over the 7 days collection period and sub-samples was taken and milled with a simple laboratory mill. The milled sub-samples were then stored in airtight bottles pending analysis.

Caecal fermentation and microbial profile

Ceacal content was collected from euthanized rabbits from subsequent incised caecum using a cotton swab stick. The total number of caecum bacteria content (*Escherichia coli*, coliform, *lactobacilli* and *streptococcus* populations) was quantifed according to document reported by American Public Health Association (Elghalid et al., 2020; Anaso and Al-hassan, 2025). Isolation of bacteria from the caecal epithelium was effectively achieved using the method as explained by the Food and Drug Administration (BAM, 1998). A reciprocal value of the dilution exponent is used to characterize the dilution factor. The units that combine to form colonies are called colony forming units, or CFU/g (Barnes and Impey, 1970). This value is stated in this way (Elghalid et al., 2020). Using the CFU technique, an incubation period of two to seven days was observed at around 30 ℃. The caecal microbiological contents of exactly 8 rabbits per treatment were gathered. On the proper selective and non-selected agar plates, the numerical quantities of anaerobic bacteria, lactose enterobacteria, and coliform bacteria were enumerated (Elghalid et al., 2020). After that, the colony count was represented in log 10 CFU in relation to 1 g of material (Abd El-Azeem et al., 2019; Anaso and Al-hassan, 2025). The caecum and ileum digesta were extracted using a gentle finger stripping technique in order to measure the pH, total volatile fatty acids (TVFAs), and ammonia-N levels (Abd El-Azeem et al., 2019). According to Said et al. (2016), the pH readings were ascertained right away. The digesta residue was separated into two halves and stored in a deep freezer at -20 °C until needed for additional analysis. The consort pH meter model P 107 with combined electrode was used to measure the pH values. The distillation method was used to estimate the ammonia-N concentration in accordance with Association of Official Analytical Chemists (AOAC, 2000). Warner (1964) noted that steam distillation was used to evaluate the quantity of the TVFA (Said et al., 2016).

Analytical methods

Feed and faecal samples of each treatment was ground to pass a 1 mm sieve in a Wiley mill and stored for further analysis. Proximate analyses of crude protein (CP), ash, ether extract (EE) and crude fibre (CF) of the experimental samples were carried in accordance with the procedures of AOAC (2000). Neutral

detergent fibre (NDF) and acid detergent fibre (ADF) were determined by the methods of Van Soest et al. (1991).

Statistical analyses

Data on nutrient digestibility, BW gain and nutrient utilization, caecal fermentation and microbial profile were subjected to variance analysis in a thoroughly randomized design with SPSS (23.0). The Duncan multiple range test was performed using the same software at a significance level of $p < 0.05$ to determine the difference between means as described and adopted from Anaso et al. (2024b); Olafadehan et al. (2023). Below is the statistical model that was employed.

$Yij = \mu + ti + eij$

Where:
Yij is the overall response to the particular parameter in question."μ," Each observation's unique overall mean, when it comes to measurable parameters, tij is the fixed effect of dietary interventions (treatments) (i = 3). For every exact estimate, eij is the random error term.

Results

Experimental diet composition

Table 2 shows the experimental diet's chemical composition.

Table 2. **Chemical composition of experimental diet**

Chemical components	% composition
Dry matter (% fresh material)	88.06
Crude protein	16.54
Ether extract	2.26
Crude fibre	13.30
Ash	9.44
Neutral detergent fibre	32.06
Acid detergent fibre	18.72
Organic matter	90.56
Nitrogen free extract	58.46

Nutrient digestibility of rabbits bucks

Table 3 shows the nutrient digestibility of rabbits supplemented with PEO in the diet. The dry matter digestibility (DMD) (60.10 – 70.98%), crude protein digestibility (CPD) (68.72 – 76.50%), crude fiber digestibility (CFD) (34.69 – 46.03%), Neutral detergent fibre digestibility (NDFD) (38.09 – 54.26%) and organic matter digestibility (OMD) (58.35-66.39%) followed the same trend with similar and higher ($p < 0.05$) digestibility in rabbits supplemented with PEO (T2 and T3) compared to the control group. Ether extract digestibility (EED) (53.92 – 73.26%) was higher ($p < 0.05$) in T3 than T1 and T2, which had similar ($p > 0.05$) digestibility. Acid detergent fibre digestibility (ADFD) (29.22 – 45.37%) was highest in T3 and lowest in T1 ($p < 0.05$) but was similar ($p > 0.05$) between T1 and T2, and T2 and T3.

Table 3. **Nutrient digestibility of rabbits supplemented PEO**

Parameter (%)	T1	T2	T3	SEM
Feed intake	26.03^{b}	27.36^{ab}	29.62^{a}	1.17
Dry matter digestibility	60.10^{b}	66.44^{a}	70.98^{a}	2.54
Crude protein digestibility	68.72^{b}	75.28^{a}	76.50^{a}	2.27
Ether extract digestibility	53.92^{b}	60.87^{b}	73.26^{a}	3.99
Crude fibre digestibility	34.69^{b}	41.85^{a}	46.03^{a}	2.83
Neutral detergent fibre	38.08^{b}	48.27^{a}	54.26^{a}	3.49
Acid detergent fibre	29.22^{b}	36.57^{ab}	45.37^{a}	4.23
Organic matter digestibility	58.35^{b}	64.08^{a}	66.39^{a}	2.31
Nitrogen free extract	43.19^{b}	51.79^{a}	57.60^{a}	2.92

^{abc} Means with the different superscripts along the row are significantly ($p < 0.05$) different
T1, 0 ml *P. thonningii* essential oil; T2, 2 ml *P. thonningii* essential oil; T3, 4 ml *P. thonningii* essential oil/kg diet.

Feed utilisation and growth performance of rabbits supplemented PEO

Table 4 shows the feed utilisation and growth parameters of animals administered PEO supplemented in diet. There were not any significant ($p > 0.05$) differences in the INBW among the three experimental groups. FBW (1096.00 – 2536.67 g), TWG (828.00 – 2275.33 g), ADG (9.90 – 27.08 g/d) and protein efficiency (2.10 – 3.99) followed the similar trend and increased with increasing level of PEO supplementation ($p < 0.05$). FCR (1.80 – 3.61) decreased with increasing level of PEO supplementation ($p < 0.05$).

Table 4. **Feed utilisation and growth performance of rabbits supplemented PEO**

Parameter	T1	T2	T3	SEM
Initial body weight (g)	268.00	259.33	261.33	56.06
Final body weight (g)	1096.69^{c}	1664^{b}	2536.67^{a}	175.93
Total weight gain (g)	828.00^{c}	1404.67^{b}	2275.33^{a}	146.44
Average daily gain (g/d)	7.89^{c}	13.38^{b}	21.67^{a}	1.40
Total feed intake (g)	2926.63^{b}	2984.95^{ab}	3162.40^{a}	92.71
Average daily feed intake	32.16^{c}	39.36^{b}	46.34^{a}	1.23
Feed conversion ratio	4.52^{a}	3.01^{b}	2.23^{c}	0.34
Protein efficiency ratio	2.10^{c}	2.90^{b}	3.99^{a}	0.25

^{abc} Means with the different superscripts along the row are significantly ($p < 0.05$) different
T1, 0 ml of *P. thonningii* essential oil; T2, 2 ml administration of *P. thonningii* essential oil; T3, 4 ml of *P. thonningii* essential oil/kg diet.

Ceacal microbial profile of rabbits supplemented PEO

Table 5 shows the ceacal microbial profile of rabbits fed PEO supplemented diet. Counts of ceacal *Escherichia coli*, coliform bacteria and *Streptococcus* species varied from 4.53 – 7.13, 3.72 – 6.42 and 1.59 – 3.01 Log_{10}CFU/g respectively with T1 having higher counts ($p < 0.05$) than T2 and T3 which had similar ($p > 0.05$) counts. Contrarily, *Lactobacillus* species (12.11 to 17.40 Log_{10}CFU/g) counts increased as the PEO supplementation level increased ($p < 0.05$).

Table 5. **Ceacal microbial profile of rabbits supplemented PEO**

Parameter (Log_{10}CFU/g)	T1	T2	T3	SEM
Escherichia coli	7.13^{a}	4.76^{b}	4.53^{b}	0.57
Coliform	6.42^{a}	3.72^{b}	4.03^{b}	0.38
Lactobacillus species	12.11^{c}	14.14^{b}	17.40^{a}	0.92
Streptococcus species	3.01^{a}	1.99^{b}	1.59^{b}	0.40

^{abc} Means with the different superscripts along the row are significantly ($p < 0.05$) different
T1, 0 ml *P. thonningii* essential oil; T2, 2 ml of *P. thonningii* essential oil; T3, 4 ml *P. thonningii* essential oil/kg diet.

Ceacal fermentation characteristics of rabbits supplemented PEO

Table 5 shows the ceacal fermentation characteristics of rabbits fed PEO supplemented diet. The ceacal pH ranged from 5.88 to 6.06 and no showed no significance ($p > 0.05$) among treatment groups. The ammonia nitrogen (16.82 – 17.61 mg/100 ml) was highest in T1 and lowest in T3 ($p < 0.05$) but was similar ($p > 0.05$) between T1 and T2, and T2 and T3.

Total volatile fatty acid (19.79 – 23.20 meq/100 ml) increased along treatment groups with lowest ($p < 0.05$) value in the control and highest in T3 (4 ml PEO). Acetic acid (13.03 – 14.80 meq/100 ml) was similar ($p > 0.05$) in rabbits supplemented with PEO which had lower concentrations ($p < 0.05$) than the control.

Butyric and propionic acid varied between 2.65 to 4.11 meq/100 ml and 0.92 to 3.25 meq/100 ml respectively. Higher and similar ($p > 0.05$) values were observed in groups supplemented with PEO, while lower ($p < 0.05$) values were seen in the control.

Table 6. **Caecal fermentation characteristics of rabbits supplemented PEO**

Parameter	T1	T2	T3	SEM
pH	6.39	5.73	5.55	0.40
Ammonia nitrogen (mg/100 ml)	17.61^{a}	17.20^{ab}	16.82^{b}	0.24
Total volatile fatty acid (meq/100 ml)	19.79^{c}	21.01^{b}	23.20^{a}	0.32
Acetic acid (meq/100 ml)	14.80^{a}	13.81^{b}	13.03^{b}	0.33
Butyric acid (meq/100 ml)	2.65^{b}	3.88^{a}	4.11^{a}	0.19
Propionic acid (meq/100 ml)	0.92^{b}	2.13^{a}	3.25^{a}	0.47

^{abc} Means with the different superscripts along the row are significantly ($p < 0.05$) different
T1, 0 ml *P. thonningii* essential oil; T2, 2 ml of *P. thonningii* essential oil; T3, 4 ml *P. thonningii* essential oil/kg diet.

Discussion

Chemical composition of experimental diet

According to Anaso et al. (2024b) and Anaso et al. (2024c), the diets' normally high DM is crucial since it indicates that there is less moisture and more nutrients. DM is a specific feed component that contains the nutrients, according to Al-Hassan and Anaso (2024) and Mowlem (1992). The experimental diet had a moisture level slightly above 10%, as shown by the DM of 88.06% fresh matter. This low moisture level is sufficient to stop mold formation, increasing the diet's

ideal shelf life (Anaso et al., 2024b). The NRC (1984) report on raising rabbits said that the CP was between 16 and 17.5%. The current study's CP of 16.54% is comparable to the 16% crude protein requirement for rabbits raised in small and medium-sized businesses (Lebas, 2013; Anaso et al., 2025).

Nutrient digestibility of rabbits fed Piliostigma thonningii essential oil supplemented diet

Digestibility for DM, CP and OM was significantly highest for experimental animals supplemented with the highest (4 ml) PEO. The improved digestibility in this study was similar to the work of El Nomeary et al. (2020), who added garlic essential oil to the maturing rabbits' basic diet and reported that it affected the CPD and CFD due to increased amounts of protein available at the cellular level for deposition in the body tissues. The result deduced this study was also researched to be similar to the findings of Patra et al. (2001); Shehata et al. (2003) and Hernandez et al. (2004) who attributed improved digestibility in their various experiments to garlic essential oil supplementation at different levels. This current result which showed an improved digestion of several nutrients, especially CP, may be due to an evenly enhanced increment in healthy and good microbes which increase the rate of stomach passage and physiologically improve food digestion by stimulating the release of bodily gastrointestinal enzymes (feed intake) (Anaso et al., 2025).

Feed utilisation and growth performance of rabbits fed Piliostigma thonningii essential oil supplemented diet

Devandra and Sere (1993) reported and implicated the plane of nutrition to clearly affect body weight gain (BWG) in domestic animals. The insignificant difference in the INBW rabbits indicates the effectiveness of randomisation of the animals which further justified the adoption of complete randomised design.

FBW, TWG and ADG were highest in treatment supplemented with 4 ml PEO. FBW was in line with the work of Omer et al. (2012) who administered Fennel (*Foeniculum vulgare*) and Oregano (*Origanum vulgare L*) EO as supplementary dietary additives improved BWG. El-Radwan and Abdel-Khalek (2007) supplemented herb mixture revealed that TWG was improved.

Feed conversion was highest in control but most efficient in treatment supplemented with 4 ml PEO. El Radwan and Abdel-Khalek (2007) and El-Nomeary et al. (2020) reported essential oils and some herbal feed additives to improve ADG and FCR for rabbits. According to El Nomeary et al. (2020), the taste and odor of the active ingredients prevent animals from consuming them, which presents a number of difficulties for the provision of herbs and essential oils to animals. Generally, a lower FCR indicates the feed to be of high potential

proving that the PEO supplemented treatment ensured efficient feed consumption, which implies better output to less input (feed). Similar to result in the current findings, Ahmed et al. (2018) reported FCR to be improved by thyme essential oil supplementation under hot environmental conditions which attributes the positive bioactive compounds on digestive efficiency leading improved FCR.

It was tentatively finalized (concluded) that the improvement reported in growth parameters could definitely be directly caused by the synergistic properties of PEO. The beta-mycerne and limonene constituent via the bioactive hexadecane, 1-methyl naphthalene and oleic acid hydrocarbons was responsible to have enhanced the body weight due to improved feed intake in animals administered the PEO. The increased weight (FBW, TWG and average daily weight gain) of T3 (with highest PEO concentration) suggests that the bioactive hydrocarbons of the PEO were effective and beneficial in enhancing the growth performance of the rabbits.

The higher FBW, TWG, ADG, FCR and PER in T3 can be adduced to the higher FI and digestibility suggesting better feed palatability and protein availability which was of benefit to the research rabbits due to PEO supplementation.

Caecal microbial profile of rabbits fed Piliostigma thonningii essential oil supplemented diet

Treatments (T2 and T3) significantly decreased the numbers of coliform bacteria, *Escherichia coli,* and *Streptococcus* species in comparison to the control group. Less coliforms were discovered in the caecum of rabbits fed a diet supplemented with 0.5 g/kg DM of thyme essential oil, according to Elghalid et al. (2020) and Placha et al. (2013). According to Elghalid et al. (2020) and Celia et al. (2016), rabbits fed a diet supplemented with a combination of essential oils, herbs, spices, and extracts known as a natural herbal formulation showed a consistent and significant decrease in bacterial divergency in their caecum. The microbial ecology of the gastrointestinal tracts, particularly the intestine, is altered by a clearly noticeable decrease in pathogenic bacteria, favoring species that are beneficial to the body (Michiels et al., 2009). This leads to an improvement in the ability of epithelial cells to regenerate intestinal villus, thereby amplifying the intestine's absorptive capacity (Mourao et al., 2006). The adhesion, colonization, and proliferation of Escherichia coli and numerous other pathogens in the digestive tracts of domestic broilers are successfully inhibited by phenolic compounds present in essential oils, herbs, and extracts (Jang et al., 2007; Simitzis, 2017). Elghalid et al. (2020) demonstrated that phytogenic extracts containing wide antimicrobial action (antibacterial, antifungal, and antiviral qualities) are present in several known EOs and aromatic plants. Numerous digestive disorders that have been connected to higher rates of morbidity and mortality in young and growing

rabbits are caused by pathogenic bacteria, which are commonly found in the intestinal tracts of rabbits (Elghalid et al., 2020).

Similar to the work of Elghalid et al. (2020), it was clearly demonstrated from the current experiment that the maximum dose of P. thoninngii EO (T3) increased the numbers of Lactobacillus spp. bacteria, which are favorable. EOs, natural herbs, and general phytogenic extracts have been shown to reduce harmful bacteria while fostering beneficial microbes such *Lactobacillus* spp. (Elghalid et al., 2020; Simitzis 2017). One prevalent and well-known resident of the rabbit intestinal microbiota that supports healthy gut activity and function is *Lactobacillus* spp. (Elghalid et al., 2020; Celia et al., 2016). By continuously generating low-molecular weight peptides that cause immunological activation, lactobacilli bacteria interact with the gut immune complex system and offer temporary protection against both simple and complex medical disorders (Muir et al., 2000). Additionally, Elghalid et al. (2020) agreed with research conducted by Rinttila and Apajalahti (2013), explaining that changing the receptors that these bacteria employ, brings about an increase in Lactobacilli bacteria thereby enhancing colonization resistance against pathogenic microorganisms.

Antibacterial properties of sesquiterpene and monoterpene hydrocarbons through bioactive hexadecanoic acid, limonene and alpha-pinene of *Piliostigma thonningii* essential oil was found to be potent against the harmful microorganisms in the current investigation.

Caecal fermentation characteristics of rabbits fed Piliostigma thonningii essential oil supplemented diet

Total volatile fatty acids (VFA), acetic acid, butyric acid, propionic acid, ammonia-N and pH values are general measures of caecum microbial activity.

According to Abd El-Azeem et al. (2019), the groups supplemented with PEO exhibit a higher level of VFA and ammonia-N in the caecum in comparison to control animals. These alterations in the fermentation pattern of VFA and ammonia-N suggest major changes in the caecum microorganisms. The observed rise in T2 and T3 levels could suggest the presence of minimal or no bacterial overgrowth, elevated metabolic bacterial activity, or reduced absorptive ability of the rabbits' gut wall (Krieg et al., 2009).

Gidenne (2006) found a favorable correlation between elevated VFA and propionic acid and antioxidant activity exhibited by phytogenics, uronic acids, and different essential oils. VFA is responsible for an extra rise in rabbits' energy needs (Bassiony et al., 2015). For enterocytes to be enhanced, particularly in the lower digestive tract, butyric acid is essential (Butzner et al., 1994). This could cause the colonocytes to experience an energy deficit and reduce their ability to absorb (Krieg et al., 2009). Moreover, I-butyric acid is thought to be a sign of elevated intestine proteloytic activity (Cardona et al., 2005), and a variation in its level

signifies a change in the metabolism of bacterial proteins. The current findings are in line with those of Krieg et al. (2009) and Bassiony et al. (2015), who employed a combination of herbal feed additives on growing rabbits.

Conclusion

In the light of the present findings, PEO supplementation improved nutrient digestibility, feed utilisation efficiency, growth performance and enhanced rabbits' caecal fermentation characteristics in the context of the current findings. Supplementing rabbits with *P. thonningii* essential oil enhanced their caecal microbial profile by decreasing the number of harmful bacteria (E. coli, Coliform, and *Streptococcus* spp.) and increasing the population of beneficial bacteria (*Lactobacillus* spp.). PEO supplementation at 4 ml/kg diet is superior to 2 ml/kg diet due to better results obtained for some parameters.

Svarljivost hranljivih materija, performanse rasta, mikrobni profil u cekumu i karakteristike fermentacije zečeva hranjenih obrokom sa dodatkom eteričnog ulja *Piliostigma thonningii*

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Rezime

Cilj ovog eksperimentalnog istraživanja bio je da se ispitaju efekti esencijalnog ulja *Piliostigma thonningii* na svarljivost hranljivih materija, karakteristike rasta, mikrobiom cekuma i karakteristike fermentacije kod zečeva. Zečevi su nasumično raspoređeni u pojedinačne grupe od po 3 jedinke, koje su obuhvatale tačno 15 zečeva po grupi, i bili su uravnoteženi po telesnoj masi tako da je prosečna početna telesna težina (TT) svake grupe bila slična - 262,89 ± 22,36 g - u potpuno randomizovanom dizajnu. Prvi tretman (T1) bio je bazalna kontrolna dijeta. U tretmanima 2 i 3, bazalna kontrolna dijeta je dopunjena sa 2 ml PEO/kg obroka i 4 ml PEO/kg obroka, respektivno. Rezultati za konačnu telesnu masu, ukupnu masu, prosečne dnevne priraste, konverziju hrane, efikasnosti proteina, *Lactobabacillus* spp. u cekumu, ukupne isparljive masne kiseline ($p< 0,05$) su poboljšani sa povećanjem nivoa suplementacije PEO. Osim svarljivosti etarskog ekstrakta i ADF svarljivosti vlakana, prividne svarljivosti suve materije i svih hranljivih materija, cekalna buterna i propionska kiselina bile su veće ($p < 0,05$) kod T2 i T3 nego kod T1. ADF svarljivost vlakana bila je veća ($P < 0,05$) kod T3 nego kod T1, pri čemu

je T2 bio na sredini ($p > 0,05$) između T1 i T3, dok je svarljivost etarskog ekstrakta bila najveća kod T3 ($p < 0,05$) i slična za T1 i T2 ($p > 0,05$). Broj *E. coli*, koliformnih bakterija i *Streptococcus* spp. u cekumu i sirćetna kiselina bili su značajno veći ($p < 0,05$) kod T1 nego kod T2 i T3. Tretmani nisu uticali na pH vrednost cekuma ($p > 0,05$). Amonijačni azot je bio veći ($p < 0,05$) kod T1 u odnosu na T3, dok je T2 bio na sredini između T1 i T3 ($p > 0,05$). Zaključeno je da je treća grupa, kojoj je dato 4 ml PEO/kg obroka, optimalan nivo suplementacije, jer je bila efikasnija u poboljšanju performansi zečeva i da je suplementacija eteričnim uljem *P. thonningii* poboljšala korišćenje hrane, svarljivost hranljivih materija, performanse rasta eksperimentalnih zečeva i smanjila broj patogenih bakterija u cekumu, čime se poboljšava zdravstveno stanje eksperimentalnih zečeva.

Ključne reči: efekti hrane, korišćenje hrane, crevne bakterije i metabolizam, fitogena hranljiva supstanca, pseudopreživari.

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

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MONITORING AND DETECTION OF GENETICALLY MODIFIED SOYBEAN IN ANIMAL FEED USING REAL-TIME PCR (2019–2024)

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Original scientific paper

Abstract: The detection and monitoring of genetically modified organisms (GMOs) in animal feed is becoming increasingly important due to legal requirements related to labeling and control of authorization for placing GM products on the EU market. This study focuses on the presence and diversity of GM sequences in soybean meal samples collected between 2019 and 2024. A total of 94 samples were analyzed using sensitive molecular methods, including qualitative and quantitative PCR (Real-time PCR), in accordance with validated international protocols. DNA extraction was carried out using the CTAB method, and GM sequences were detected using specific primers for the most common promoters, terminators, and transgenes, including P-35S, T-nos, GTS 40-3-2, and A5547-127. The analysis was further improved by the use of the GMO Matrix tool and reference materials for result validation. The results showed a clear trend of increasing numbers of analyzed samples and greater diversity of detected GM sequences during the observed period, especially in 2023 and 2024. Although the GTS 40-3-2 sequence remained the most frequently detected, more complex combinations of transgenes such as Cry1Ab/Ac and CTP2-CP4-EPSPS, along with Pat and Bar genes, were also identified. These findings indicate the presence of modified soybean lines MON87701, MON877051-7, DAS44406, DAS81419-2, and MON87708-9, pointing to a growing diversity of GM traits in animal feed. These results highlight the importance of continuous GMO monitoring in animal feed, as well as the necessity of maintaining high standards of analytical accuracy. Systematic testing contributes to the safety of the food chain, the protection of animal and human health, and sustainable food production.

Key words: GMO, soybean meal, real-time PCR, animal feed, GMO monitoring

Introduction

Genetically modified organisms (GMOs) have become an integral component of modern agriculture, offering benefits such as improved crop yields, pest resistance, and herbicide tolerance. However, their widespread adoption has raised significant concerns regarding food and feed safety, environmental impacts, and consumer acceptance. In the European Union (EU), the regulation of GMOs is particularly stringent, requiring rigorous risk assessments and traceability systems to ensure transparency and safeguard public health (European Commission, 2021; EFSA, 2018).

Soybean (*Glycine max*) is one of the most commonly genetically modified crops globally, with more than 75% of global soybean cultivation attributed to GM varieties, predominantly those expressing herbicide tolerance traits such as CP4 EPSPS or stacked events combining multiple traits. Due to its high protein content, soybean meal is widely used as a primary protein source in livestock diets (OECD/FAO, 2021). Consequently, the presence of genetically modified soybean in animal feed has become a focal point for food and feed control authorities, particularly in regions where strict labelling and authorization requirements apply, such as the EU (Holst-Jensen et al., 2012; Mazzara et al., 2007).

The accurate detection and identification of GMOs in food and feed products are essential for regulatory compliance, risk management, and meeting consumer demand for transparency (Bawa and Anilakumar, 2013; Fraiture et al., 2015). Molecular techniques such as the polymerase chain reaction (PCR), including Real-Time PCR and quantitative PCR (qPCR), have become standard tools in GMO testing due to their high specificity, sensitivity, and reproducibility (Mazzara et al., 2007). These techniques enable the detection of common genetic elements (e.g., promoter P-35S, terminator T-nos), event-specific sequences, and quantification of GM material in complex food and feed matrices (Holst-Jensen et al., 2012).

Despite the routine use of PCR-based methods in official control laboratories, limited data are available on the longitudinal trends of GMO presence in feed ingredients, particularly in the context of temporal changes in the market, regulatory approvals, and trade patterns. Monitoring these trends is vital for assessing compliance with legal thresholds, tracking the introduction of new GM events, and informing risk-based sampling strategies (Grohmann et al., 2019).

In this study, PCR-based methods were applied to detect and identify GM sequences in soybean meal samples collected over a six-year period (2019–2024). Using validated detection protocols, certified reference materials (CRMs), and up-to-date sequence databases. Particular attention was given to the accuracy and sensitivity of the analytical procedures, as well as to characterizing the diversity of GM events identified in the tested samples. The primary objective was to evaluate the frequency and diversity of GM sequences in soybean meal entering the feed

supply chain, with a focus on temporal trends and changes in the composition of detected genetic modifications over the study period.

Materials and Methods

For the detection and confirmation of the presence of genetically modified organisms (GMOs) in food, seed materials and animal feed various analytical methods are employed, with Real Time polymerase chain reaction (RT-PCR) being the most commonly used. This technique enables the specific identification of transgenic DNA sequences and, in the case of a quantitative approach, the determination of the proportion of genetically modified lines within a sample (Dong et al., 2008). Samples of animal feed intended for analysis were collected between early 2019 and the end of 2024. Animal feed samples were received at the Central Laboratory for Seed Testing and Biotechnological Analysis, in the Department for Biotechnological Analysis of the Croatian Agency for Agriculture and Food (CSR, HAPIH) as part of official controls. DNA extraction was performed following the CTAB protocol (Lipp et al., 1999; Querci et al., 2020). After homogenization, 200 mg of each sample was weighed and mixed with 1000 µL of CTAB extraction buffer preheated to 65 °C. Following cell lysis, DNA was purified using 500 µL of chloroform. After centrifugation and phase separation, two volumes of CTAB precipitation solution were added to the aqueous phase. The DNA pellet obtained from the initial precipitation was dissolved in 350 µL of 1.2 M NaCl solution and further purified with 350 µL of chloroform. In accordance with the protocol, two precipitation steps were conducted, with the second step involving isopropanol in a volume ranging from 0.6 to 1× the sample volume. Finally, the purified DNA was washed with 70% ethanol and resuspended in 100 µL of sterile deionized water. The concentration and purity of the isolated DNA were determined spectrophotometrically (BioPhotometer, Eppendorf) by measuring absorbance at 260 nm and 280 nm (A260/A280 ratio). All DNA samples used for PCR analysis were normalized to a concentration of 50 ng/µL. This protocol is widely recognized for its reliability and efficiency in molecular detection of GMOs, particularly in complex matrices such as feed products (European Commission, 2015). The initial phase of the analysis involved qualitative screening using Real-Time PCR to detect commonly occurring genetic elements indicative of modification. All PCR reactions were conducted on the Applied Biosystems 7300 Real-Time PCR system. Detection of GMO-specific DNA sequences was performed using quantitative PCR (qPCR) with TaqMan chemistry, following the methodology described by Navarro et al. (2015). For the identification of genetic modifications, widely adopted primer sets such as P35S and T-nos were employed to target regulatory regions like promoters and terminators. Additionally, primers specific to Cry1Ab/Ac, CTP2-CP4-EPSPS, T-E9, and PAT were utilized to amplify regions associated with inserted transgenes. The integrity of DNA samples was verified using soybean-specific primers, as

described by Mazzara et al. (2007). The robustness and sensitivity of PCR-based methods have made them the gold standard in the regulatory monitoring of genetically modified materials (Grohmann et al., 2009). To identify specific genetically modified soybean events within the samples, the GMO Matrix tool was applied (Bonfini, 2023). This computational system integrates data from the GMOMETHODS database (Dong et al., 2008; Bonfini, 2012) and the Central Information System for Basic DNA Sequences (Fraiture et al., 2015), and provides validated reference methods for GMO detection and identification. The use of such databases significantly enhances accuracy and traceability in official GMO testing laboratories (Holst-Jensen et al., 2012). Certified reference materials (CRMs) of genetically modified soybean (IRMM, Geel, Belgium), with defined GMO content, were used as positive controls. The established limit of quantification for the assay was 0.1%. Each qPCR reaction was carried out in a final volume of 50 µL, comprising 40 µL of reaction mix (including Mastermix) and 10 µL of DNA sample at a concentration of 50 ng. The thermal cycling conditions were as follows: 2 minutes at 50 °C, initial denaturation at 95 °C for 10 minutes, and 45 amplification cycles of 95 °C for 15 seconds followed by 60 °C for 1 minute, where the extension step occurs. Quantification was based on co-amplification of the target GMO-specific sequence along with a standard of known concentration within the same reaction. Based on positive screening results, specific GM soybean lines were confirmed through the detection of characteristic genetic elements such as CTP2-CP4-EPSPS and T-E9, which are associated with five authorized GM soybean events.

Results and Discussion

The Table 1 presents the frequency and percentage of analysed soybean meal samples by sampling year (from the year 2019 till the year 2024), including cumulative values. The data show that the number of samples analysed was relatively low in the period from 2019 to 2021. During these three years, annual sample shares ranged from only 4.26% in 2021 to 11.70% in 2020. Altogether, only 26.60% of all samples were analysed in this initial period, indicating a limited scope of analysis, possibly due to organizational constraints, reduced demand, or limited laboratory capacity during those years.

From 2022 onwards, there is a noticeable increase in the number of analysed samples. In 2022, the number rose to 15 samples (15.96%), followed by a further increase in 2023, with 20 samples (21.28%), and reaching a peak in 2024 with 34 samples analysed, which represents the largest share at 36.17% of the total. This rising trend strongly suggests a growing interest in the quality control and monitoring of soybean meal in recent years. The increased sample frequency may be attributed to the expanded use of soybean meal as a protein-rich feed ingredient, stricter quality assurance requirements, or improvements in laboratory infrastructure and analytical procedures.

Table 1. Frequency and percentage of analysed soybeans meal depending on the sampling year

Sampling year	Frequency, N	Percent, %	Cumulative frequency, N	Cumulative percent, %
2019	10	10.64	10	10.64
2020	11	11.70	21	22.34
2021	4	4.26	25	26.60
2022	15	15.96	40	42.44
2023	20	21.28	60	63.83
2024	34	36.17	94	100.00

Cumulative data reinforce this pattern. By the end of the year 2022, a total of 42.44% of all samples had been analyzed, while in just the last two years, 2023 and 2024, more than half of the total samples (57.56%) were examined. This distribution clearly highlights the importance of recent years in terms of data volume and underlines the increased relevance of soybean meal analysis in current livestock nutrition and feed quality assurance practices.

Furthermore, in the context of animal nutrition, such an increase in soybean meal analysis is particularly relevant. Soybean meal is one of the most important plant-based protein sources used in livestock feeding, and its composition can significantly influence animal performance, nitrogen utilization efficiency, and overall farm sustainability. The observed rise in analytical frequency likely corresponds with an increased need for precise formulation of rations, especially in systems aiming to optimize protein efficiency and reduce nitrogen emissions.

Also, the increase in recent years reflects not only growing scientific and industry interest in feed quality but also broader shifts toward more data-driven, sustainable, and quality-assured animal nutrition practices. This trend underscores the importance of ongoing monitoring and reinforces the role of laboratory analysis in supporting evidence-based decision-making in livestock production systems.

Table 2 presents the identification of genetically modified (GM) sequences in analysed soybean meal samples, with a breakdown by year of sampling. The data include the number of analysed samples, the sequences that were requested for detection, the sequences that were actually detected, and the number of samples in which these sequences were found.

In 2019, 10 samples were analysed, and several GM sequences were requested, including P-35S, t-NOS, GTS 40-3-2, and others. A total of 8 samples (80%) tested positive for the sequence P-35S, T-nos, GTS 40-3-2, A5547-127, and two samples (20%) were found to contain the sequence P-35S, T-nos, GTS 40-3-2. This indicates a significant presence of genetically modified material in the samples, with the most frequent sequences being related to the GTS 40-3-2 event.

In 2020, a similar number of samples were analysed (10), with multiple GM sequences tested, including the same ones as in 2019. Five samples (50%) were detected with the sequence P-35S, T-nos, GTS 40-3-2, A5547-127, while the remaining samples tested positive for P-35S, T-nos, GTS 40-3-2. This shows a continued presence of GM material, with a higher detection rate for one specific sequence, P-35S, T-nos, GTS 40-3-2, A5547-127. In addition, a single sample in 2020 (from a different set) was tested for a broader set of sequences, including CTP2-CP4-EPSPS, Cry1Ab/Ac, and other GM markers. However, no sequences were detected in this sample, indicating that not all samples contain detectable GM material.

In 2021, only four samples were analysed, and all of them contained the sequence P-35S, T-nos, GTS 40-3-2, suggesting a high consistency in the presence of this specific GM sequence. This result shows that, although fewer samples were analysed, the prevalence of the GM sequence was still significant.

By 2022, the number of analysed samples increased to 15. The majority of the samples (14 out of 15) tested positive for the P-35S, T-nos, GTS 40-3-2, sequence, while 1 sample contained P-35S, T-nos, GTS 40-3-2, A5547-127. This highlights the continued presence of genetically modified sequences, with a slight increase in the diversity of sequences detected in 2022 compared to previous years.

In 2023, 18 samples were analysed, one sample (approximately 5%) tested positive for P-35S, T-nos, GTS 40-3-2, A5547-127, while 17 samples (94%) contained P-35S, T-nos, GTS 40-3-2. Additionally, two more samples were analysed with a broader range of GM sequences, including CTP2-CP4-EPSPS, Cry1Ab/Ac, and others, and both tested positive for several GM markers. These results demonstrate an even higher frequency of genetically modified material in the samples, with an increasing diversity of sequences detected in 2023.

The data for 2024 shows a continued trend with 32 analysed samples. Among these, 22 samples (68.75%) tested positive for P-35S, T-nos, GTS 40-3-2, and 10 samples (31.25%) were found to contain the sequence P-35S, T-nos, GTS 40-3-2, A5547-127. Additionally, two samples analysed in 2024 tested positive for a more complex set of GM sequences, including CP4-EPSPS, Cry1Ab/Ac, and others, indicating that the genetic modification in these soybean meal samples is becoming increasingly diversified.

Finally, the results from Table 2 reveal a consistent presence of genetically modified sequences across all years, with a notable increase in the detection of more diverse GM sequences over time. While the most common sequences remained related to the GTS 40-3-2 event (P-35S, T-nos, GTS 40-3-2), newer sequences and more complex combinations of genetic modifications started to appear, particularly from 2023 onwards. This trend suggests that the genetic modification in soybean meal is becoming more varied, possibly reflecting changes in the sources of the soybean meal or in the adoption of new GM crops.

Table 2. Presentation of identified genetically modified sequences in analysed soybeans meal depending on the sampling year

Sampling year	N, analysed samples	Requested sequences	Detected sequences	N, detected samples
2019	10	P-35S, t-NOS, GTS 40-3-2, DP-356043-5, A2704-12, A5547-127, FG 72	P-35S, T-nos, GTS 40-3-2, A5547-127,	8
			P-35S, T-nos, GTS 40-3-2,	2
2020	10	P-35S, t-NOS, GTS 40-3-2, DP-356043-5, A2704-12, A5547-127, FG 72	P-35S, T-nos, GTS 40-3-2, A5547-127,	5
			P-35S, T-nos, GTS 40-3-2,	5
2020	1	P-35S, t-NOS, CP4-EPSPS, Cry1Ab/Ac, PAT, MON89788, GTS 40-3-2, MON87701, MON 87705, 356043, A5547-127, A2704-12, DAS-44406-6	/	0
2021	4	P-35S, t-NOS, GTS 40-3-2, DP-356043-5, A2704-12, A5547-127, FG 72	P-35S, T-nos, GTS 40-3-2,	4
2022	15	P-35S, t-NOS, GTS 40-3-2, DP-356043-5, A2704-12, A5547-127, FG 72	P-35S, T-nos, GTS 40-3-2,	14
			P-35S, T-nos, GTS 40-3-2, A5547-127,	1
2023	18	P-35S, t-NOS, GTS 40-3-2, DP-356043-5, A2704-12, A5547-127, FG 72	P-35S, T-nos, GTS 40-3-2, A5547-127,	1
			P-35S, T-nos, GTS 40-3-2,	17
	2	P-35S, t-NOS, CTP2-CP4-EPSPS, Cry1Ab/Ac, PAT, Bar, tE9, MON89788, GTS 40-3-2, DP-356043-5, DAS 44406, MON87701, DAS 81419-2, DAS-68416-4, MON87705, FG72, MON87751-7, MON87708-9, MON87769-7	MON89788, DAS 44406, MON87701, DAS 81419-2, MON87751-7, MON87708-9	1
			CTP2-CP4 EPSPS, Cry1Ab/Ac, PAT, tE9, MON89788, MON87701, DAS 81419-2, MON87751-7, MON87708-9	1
2024	32	P-35S, t-NOS, GTS 40-3-2, DP-356043-5, A2704-12, A5547-127, FG 72	P-35S, T-nos, GTS 40-3-2,	22
			P-35S, T-nos, GTS 40-3-2, A5547-127,	10
	2	P-35S, t-NOS, CTP2-CP4 EPSPS, Cry1Ab/Ac, PAT, Bar, tE9, MON89788, GTS 40-3-2, DP-356043-5, DAS 44406, MON87701, A2704-12, DAS 81419-2, DAS-68416-4, MON87705, A5547-127, FG72, MON87751-7, MON87708-9, BPS-CV127-9, MON87769-7	CTP2-CP4 EPSPS, Cry1Ab/Ac, tE9, MON89788, MON87701, MON87751-7, MON87708-9	1
			CTP2-CP4 EPSPS, Cry1Ab/Ac, PAT, tE9, MON89788, DAS 44406, MON87701, MON87751-7, MON87708-9	1

Conclusion

The obtained results indicate a clear trend toward increased monitoring of soybean meal quality and genetic composition over the years. The frequency of analysed samples has risen significantly, especially in the last two years, reflecting a growing focus on feed safety, sustainability, and regulatory compliance in animal nutrition. This shift suggests a heightened awareness of the importance of feed quality, driven by evolving industry standards and consumer demands.

Simultaneously, the detection of genetically modified (GM) sequences in soybean meal has become more widespread, with a marked increase in the variety of GM traits identified over time. While the GTS 40-3-2 event was the most commonly detected sequence, new and more complex GM sequences have emerged, indicating that genetically modified soybeans are becoming more diverse in their traits. This diversification is likely a result of ongoing developments in biotechnology and the increasing use of genetically modified crops in agricultural production.

These findings highlight the need for continued monitoring and regulation of genetically modified materials in animal feed. As the presence and diversity of GM traits in soybean meal grow, it is essential to assess their potential impact on animal health, nutrition, and food safety. The results also underscore the importance of adapting regulatory frameworks and testing protocols to ensure the safe and responsible use of genetically modified ingredients in livestock feed, supporting sustainable and traceable food production systems.

Praćenje i otkrivanje genetski modifikovane soje u hrani za životinje pomoću Real time PCR-a (2019 – 2024)

Katarina Pandžić, Zvonimir Steiner, Čedomir Radović, Ksenija Duka, Vesna Gantner

Rezime

Otkrivanje i praćenje genetski modifikovanih organizama (GMO) u stočnoj hrani postaje sve važnije zbog zakonskih zahteva u pogledu označavanja i kontrole autorizacije modifikacija za stavljanje na tržište EU. Ovo istraživanje usmereno je na prisutnost i raznolikost GM sekvenci u uzorcima sojine sačme prikupljenima u periodu od 2019. do 2024. godine. Ukupno je analizirano 94 uzorka primenom osetljivih molekularnih metoda, uključujući kvalitativni i kvantitativni RT-PCR (Real-time PCR), u skladu s validiranim međunarodnim protokolima. Ekstrakcija DNK sprovedena je prema CTAB metodi, a detekcija GM sekvenci izvršena je

pomoću specifičnih prajmera za najčešće promotore, terminatore i transgene, uključujući P-35S, T-nos, GTS 40-3-2 i A5547-127. Analiza je dodatno unapređena korišćenjem alata GMO Matrix i referentnih materijala za validaciju rezultata. Rezultati su pokazali jasan trend porasta broja analiziranih uzoraka te raznolikosti detektovanih GM sekvenci tokom posmatranog perioda, naročito u 2023. i 2024. godini. Iako je sekvenca GTS 40-3-2 ostala najčešće detektovana, pojavile su se i kompleksnije kombinacije transgena poput Cry1Ab/Ac i CTP2-CP4-EPSPS, Pat i Bar gena koji ukazuju na prisutnost modifikovanih linija soje MON87701, MON877051-7, DAS44406, DAS81419-2, MON87708-9 i upućuje na raznolikosti GM svojstava u stočnoj hrani. Ovi rezultati naglašavaju važnost kontinuiranog praćenja GMO-a u stočnoj hrani, kao i nužnost održavanja visokih standarda analitičke tačnosti. Sistematsko testiranje doprinosi sigurnosti hranidbenog lanca, zaštiti zdravlja životinja i ljudi, te održivoj proizvodnji hrane.

Ključne reči: GMO, sojina sačma, real-time PCR, stočna hrana, GMO monitoring

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

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INFLUENCE OF STORED BUCKWHEAT ON COLOUR PARAMETERS OF EMULSION-TYPE CHICKEN SAUSAGES

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Abstract: The standard emulsion-type chicken sausage formulation was modified by incorporating buckwheat flour stored 0, 3, 6 and 9 months to obtain an enriched product and monitor the effect of instrumental colour change. The control (CON) and four combined frankfurter-buckwheat products with time-determined stored flour (FB0, FB3, FB6, and FB9) were analysed for instrumental colour properties (L*, a*, b*, *C**, *h*, *S*, ΔE). The results were statistically processed by one-way ANOVA ($P<0.05$) and Tukey's HSD post-hoc tests ($P<0.05$). Added buckwheat flour slightly affects L*, and significantly a* and b*, with the highest values in FB0 (similar to CON), which was lighter, redder and yellower than the other combined products. The *C** value significantly decreased ($P<0.05$), while the *h* value significantly increased ($P<0.05$) which caused lower chromatic purity and a more yellow nuance in FB9 (20.65, 45.40). Significant decrease in the *S* value resulted in the greatest attenuation in overall colour perception at FB6, and FB9. An increase trend of ΔE was observed, indicating the differentiation of sausages with respect to effect on total colour ($\Delta E \leq 5.08$). The results suggest that emulsion-type chicken sausages enriched with stored buckwheat flour are technologically justified and colour-sensory acceptable as end-products for consumers.

Key words: instrumental colour, buckwheat, chicken sausage, enriched product

Introduction

In recent years, with a focus on nutritionally justifiable and health-beneficial food, the attention has been directed to functional foods enriched with proven bioactive ingredients. In the modern meat industry, the addition of plant-based raw materials (grains, flour, husks, powder, extracts) into meat product formulations can effectively enhance various chemical, nutritional, functional, technological, and sensory properties (Safar et al., 2022; Yessengaziyeva et al., 2023). For these reasons, the development of recipes led to the production of composite products similar to frankfurter-type sausages, semi-smoked sausages, pork meatballs, horsemeat patties, chicken patties (Muchekeza et al., 2021; Salejda et al., 2022).

Buckwheat and its products are recognized for their health benefits due to high nutritional and functional value (Salejda et al., 2022; Coţovanu et al., 2023). It is characterized by the high content of bioflavonoids and minerals (K, B, Fe, Cu, Cr, Zn, Co, Ni, Ca, Mg, P, Na), vitamins B and E, as well as dietary fiber, essential amino acids and polyunsaturated fatty acids (Lee et al., 1995; Baumgertel et al., 2003; Salejda et al., 2022). As a staple food in the human diet, buckwheat grains are often stored for a prolonged period due to strategic reasons.

On the other hand, emulsion-type meat products such as frankfurter, are very popular and consumed worldwide (Wagh et al., 2015). A typical frankfurter consists of 40% pork and 60% beef, although it can also be made from 100% pork, 100% beef, and 100% poultry meat, as well as a combination of these types of meat. Another important raw material is fat tissue, which, together with meat, has a significant impact on yield, texture and microstructure (Mittal, 2005). Non-meat ingredients such as water, salt, nitrites, sugars, ascorbates, and phosphates contribute to the quality, taste, and flavor, while spices, flavorings, and antioxidants significantly influence the flavor profile (Lonergan et al., 2019).

Generally, meat products are considered unhealthy due to their high lipid and cholesterol content. However, incorporating buckwheat and its products in well-known conventional meat products, among others, frankfurters, enables the development of functional meat foods with improved quality. Despite their favorable nutritional profile, buckwheat-enriched meat products may exhibit altered sensory characteristics that negatively influence consumer acceptance (Pietrzak et al., 2022). Therefore, less favourable sensory features, specifically colour, may reduce the quality attributes of overall acceptability for consumers. These colour changes can be even greater, i.e. enhanced, as green buckwheat flour is used in the production of emulsion-type sausages (e.g. frankfurter-type) that require thermal processing (Wagh et al., 2015; Atambayeva et al., 2023; Yessengaziyeva et al., 2023; Rakić et al., 2024). Some factors, such as oxidative

reactions, the Maillard reaction, product drying, and the presence of antioxidants, are directly related to the colour change of frankfurters (Wagh et al., 2015).

The aim of this study was to evaluate the colorimetric (L*, a*, b*, *C**, *h*, *S*, and ΔE) characteristics and provide a technological reference for colour changes based on the incorporation of 0-, 3-, 6- and 9-months stored buckwheat flour in the formulation of emulsion-type chicken sausages to obtain technologically justified and colour-sensory acceptable combined end-products for consumers.

Materials and Methods

Plant material

The buckwheat variety used in this study was "*Novosadska*", which was harvested at the seed's technological maturity. Sampling was carried out according to ISO 24333 (2009), and a bulk of 2 kg sample was stored as previously reported Ping-Ping et al. (2019). Briefly, grains were equally distributed in eight samples, of which six were stored in plastic containers for 3, 6, and 9 months in a drying oven (Digitheat-TFT, J.P Selecta, Barcelona, Spain). The conditions were maintained at a temperature of 40 ± 2 °C with thermoregulation control and a relative humidity of 50%. At the end of each testing period, a subsample (approximately 0.5 kg) was formed from two samples, crushed and ground at a speed of 20000 rpm in an A10 laboratory mill (IKA Works Inc., NC, Wilmington, USA). A wholegrain buckwheat flour material was prepared in triplicate (particle size of 1 mm), and used for the preparation of emulsion-type chicken sausages.

Emulsion-type chicken sausage preparation

Emulsion-type chicken sausages were prepared in a meat processing plant "Milan" located in Dobanovci, Belgrade, which specializes in the processing and sale of chicken meat and products. Five treatments of sausages were prepared, each in batches of 2 kg, with the formulations shown in Table 1, according to Lee et al. (2018), with some modifications. The production process was common for industrial production and identical for all treatments. All formulations contained equal amounts of ingredients, except for the addition of buckwheat flour. The control treatment (CON) was made of mechanically separated chicken meat (MSM) without adding buckwheat flour, while in other treatments, buckwheat flour stored for 0 (FB0), 3 (FB3), 6 (FB6), and 9 (FB9) months was separately added. After preparation, emulsion-type chicken sausages were vacuum-packed (with 99.9% of vacuum level) and stored at 2 ± 2 °C before analysis. Three replications of the experiment were conducted.

Table 1. Recipe formulation of emulsion-type chicken sausages (kg)

Composition (kg)/Group	CON	FB0	FB3	FB6	FB9
MSM	1.13	1.07	1.07	1.07	1.07
Buckwheat flour	/	0.06	0.06	0.06	0.06
Chicken skin	0.38	0.38	0.38	0.38	0.38
Ice	0.38	0.38	0.38	0.38	0.38
Ingredients	0.11	0.11	0.11	0.11	0.11

Note: **Ingredients:** 2.00% potato starch, 1.80% salt (with 0.5% $NaNO_2$), 0.85% seasoning, 0.80% beef protein, 0.30% stabilizer - complete, 0.10% carrageenan, and 0.01% colour (E120).

Determination of instrumental colour parameters

The instrumental colour was measured at five opposite points of the longitudinal section for each emulsion-type chicken sausage on chromameter CR-400 (Konica Minolta Sensing Inc., Osaka, Japan). Conditions were corresponded to diffuse light D-65 (average daylight which has a correlated colour temperature of approx. 6 500 K), a standard angle of 2 degrees of shelter and 8 mm aperture of the measuring head as described by Stajić et al. (2014).

The results were expressed in CIEL*a*b* system (CIE, 1976) as L* (psychometer light), a* (psychometer tone) and b* (psychometer chroma), where L* value indicates the lightness from black (0) to white (100), a* value varies from green (-) to red (+), and b* value ranges from blue (-) to yellow (+). Before measurement, chromameter was calibrated against a white standard (tile) (L* = +97.83, a* = –0.43, b* = +1.98).

*C** (chroma - percentage of black, white or gray; eq. 1) and *h* (hue angle; 0° - red colour, 90° - yellow colour; eq. 2) were calculated using the following equations:

$$C^* = \sqrt{(a^*)^2 + (b^*)^2} \quad \text{(eq. 1)}$$

$$h = \tan^{-1}\left(\frac{b^*}{a^*}\right) \quad \text{(eq. 2)}$$

S (perceived saturation; eq. 3) defined as the proportion of pure chromatic colour in the total colour sensation was calculated using the following equation:

$$S = \frac{C^*}{\sqrt{C^{*2}+L^{*2}}}\ 100\% \quad \text{(eq. 3)}$$

Total colour difference (ΔE; eq. 4) of treatments with incorporated buckwheat flour (stored for 0, 3, 6 and 9 months) relative to CON (without buckwheat flour) was calculated as follows (Pietrzak et al., 2022):

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$ (eq. 4)

Statistical analysis

The instrumental colour parameters (mean (M) ± standard deviation (SD)) were analysed by one-factor analysis of variance (one-way ANOVA, $P<0.05$) to evaluate the effect of sausage formulation. Tukey's HSD (Honestly Significant Difference, $P<0.05$) post-hoc test was applied to identify significant differences between means. Statistical analyzes were performed using Statistica 12.5 software (StatSoft, Inc., Tulsa, OK, USA).

Results and Discussion

Table 2 shows the values of instrumental colour parameters in relation to different emulsion-type chicken sausages as a product type factor. Different formulations of the same sausage product had a statistically significant influence on all instrumental colour parameters ($P<0.05$). Moreover, progressive changes were noted with increasing storage time of buckwheat flour, in all observed instrumental colour parameters.

Table 2. Changes in instrumental colour parameters in relation to the storage period of buckwheat flour added to emulsion-type chicken sausages

n=3 (five points each)	Product type				
Parameters	CON	FB0	FB3	FB6	FB9
L*	59.25±0.499^{ab}	60.65±0.467^{b}	58.70±0.091^{a}	58.95±0.523^{a}	58.39±0.876^{a}
a*	19.07±0.316^{c}	18.57±0.190^{c}	15.86±0.092^{b}	15.09±0.361^{a}	14.50±0.170^{a}
b*	16.48±0.454^{b}	16.25±0.212^{b}	15.22±0.127^{a}	14.96±0.285^{a}	14.71±0.112^{a}
*C**	25.21±0.537^{c}	24.67±0.275^{c}	21.98±0.147^{b}	21.25±0.456^{ab}	20.65±0.193^{a}
h	40.83±0.307^{a}	41.18±0.159^{a}	43.82±0.101^{b}	44.75±0.139^{c}	45.40±0.157^{d}
S	39.14±0.826^{d}	37.68±0.304^{c}	35.06±0.181^{b}	33.91±0.647^{ab}	33.35±0.259^{a}
ΔE	-	1.53±0.923^{a}	3.53±0.365^{b}	4.29±0.198^{bc}	5.08±0.419^{c}

$^{a, b, c, d}$ Means within the same row with different superscripts differ significantly ($P<0.05$); Emulsion-type chicken sausages: CON: without buckwheat flour; FB0: 3% of buckwheat flour at 0 months of storage; FB3: 3% of buckwheat flour at 3 months of storage; FB6: 3% of buckwheat flour at 6 months of storage; FB9: 3% of buckwheat flour at 9 months of storage.

Compared to the CON product, the storage period of buckwheat incorporated into the products did not affect the L* values ($P>0.05$). However, emulsion-type chicken sausage with 0 months stored buckwheat flour (FB0) was

significantly lighter in comparison to other composite products. This difference in lightness (darkening) was associated with buckwheat grains (flour) stored for 3-, 6- and 9-months as development of lipid oxidation and the Maillard reaction products readily occurs during thermally assisted long-term storage and drying (Jambrec et al., 2011; Pietrzak et al., 2022; Pisinov et al., 2024). Generally, the findings of Lee et al. (2018) and Kilincceker and Karahan (2020) were in agreement with our results (although FB0 to CON is not significant), as they found that added buckwheat flour to emulsion-type pork sausages (3%) or chicken meatballs caused an increase in L* values. Later disagreements with our results (darker FB3, FB6, FB9 *cf.* CON) came from the fact that their research was performed with only one, non-stored type of buckwheat flour.

In relation to the CON product, significant differences in a* and b* values were observed in FB3, as well as in FB6 and FB9. By adding buckwheat grains (flour) stored for a longer period, 3-9 months, red and yellow tones of emulsion-type chicken sausages progressively decreased (from 15.86 to 14.50, and from 15.22 to 14.71, respectively). These decreasing trends could be explained by the loss of phenolic compounds as natural pigments, which is even more pronounced during thermally assisted long-term storage and drying (Kilincceker and Karahan, 2020; Pisinov et al., 2024). Regarding a* and b* values, our results were in agreement with the findings of Kilincceker and Karahan (2020), who found that added buckwheat flour caused a decrease in redness and yellowness of fried chicken meatballs. Similarly, Lee et al. (2018) reported that adding 3% of buckwheat powder in emulsion-type pork sausage resulted in a lower a* value, which was in accordance with our results, especially for FB3, FB6, and FB9. However, there is disagreement about the b* value, which could be explained by the source, form, and amount of flour added to the product.

The significantly lower C^* values ($P<0.05$) compared to CON, were found in emulsion-type chicken sausage with incorporated 3-month stored buckwheat flour (FB3) and more long-term stored buckwheat flour (FB6 and FB9). Increasing the storage time of buckwheat flour included in the formulation resulted in FB9 sausages having lower chromatic purity, showing the smallest distance from lightness compared to other sausages. Similar to chroma (C^*), hue angle (h) values between CON and FB0 did not differ significantly, but the significance increased with FB3, as well as in FB6 and FB9. The addition of buckwheat grains (flour) stored from 0- to 9-months, led to a significant progressive increase in the proportion of yellow nuance in emulsion-type chicken sausage, with the largest change in value at FB9. Cavalheiro et al. (2013) stated that C^* and h values depend on a* and b* values, and changes in these values are caused by the same reasons as those in a* and b*. Therefore, the progressive loss of phenolic compounds was more pronounced as storage time of buckwheat flour increases from 0- to 9-months, which led to less saturated sausage products because a* and b* decreased, and caused changes from red to yellow as the decrease of a* was more emphasized

than that of b*. Varga-Visi et al. (2021) came to the same conclusion by examining sausage with paprika, where the changes in colour were attributed to the deterioration of the chromophores in meat and paprika, i.e. the decomposition of those pigments.

The *S* values were significantly different ($P<0.05$) for all emulsion-type chicken sausages compared to CON, which had the highest value. By adding long-term stored buckwheat flour into the formulation, a decreasing trend of saturation was observed, which became more pronounced with longer buckwheat storage. Our results showed that the proportion of pure chromatic colour in overall colour sensation was significantly reduced from CON to FB9. Since the *S* value is derived from L* and *C** values, their reduction by incorporating long-stored buckwheat flour into the formulation directly causes a decreasing trend in *S* value from CON to FB9, for the same reasons as those for L* and *C**.

Further, total colour difference (ΔE) values gradually increased with the addition of longer-stored buckwheat flour to the formulation. The lowest ΔE value was observed for FB0, and the highest for FB9, while there was no significant difference between FB3 and FB6 values ($P>0.05$). Variation in L*, a* and b* colour parameters of emulsion-type chicken sausages made with differently stored buckwheat flour affected the total colour difference parameter (ΔE). This means that compared to CON, the highest degree of colour change was observed for FB9, i.e. emulsion-type chicken sausage whose formulation contains buckwheat flour stored for 9 months. It is known that $\Delta E<2.0$ values indicate a colour difference compared to the control that cannot be detected by the human eye (Pietrzak et al., 2022). Wójtowicz et al. (2013) investigated colour parameters of extruded corn snacks enriched with buckwheat flour up to 50%, and found colour differences between treatments similar to our study. In contrast to our results, Pietrzak et al. (2022) reported that there was no significant difference in ΔE values when testing the effect of buckwheat hull extracts on the quality of chicken meatballs.

Conclusion

The results indicated the potential utilization of long-term stored buckwheat grains (flour) as a functional ingredient and valuable plant-based raw material in the production of enriched, combined products such as vacuum-packed emulsion-type chicken sausages. The addition of 3% buckwheat flour stored for 0-, 3-, 6- and 9-months in the formulation of emulsion-type chicken sausages slightly decreased lightness (L*), but significantly lowered a* and b* values. Furthermore, instrumental colour analysis showed significantly decreased *C** and *S* values, while the *h* value progressively increased. Also, the addition of stored buckwheat flour resulted in differentiation of sausages in terms of the impact on total colour ($\Delta E \leq 5.08$). The degree of correlation between instrumental colour values and combined

products depends on all factors governing lipid oxidation, the Maillard reaction and levels of phenolic compounds. Considering that the instrumental colour represents only a part of the more complex methodology of the product sensory evaluation, it is necessary to conduct more extensive researches of sensory attributes (taste, odour...), textural profile, and the technological properties of the buckwheat-enriched meat emulsion stability. Since colour changes mainly influence purchasing decisions, emulsion-type chicken sausages enriched with stored buckwheat flour proved to be technologically viable and colour-sensory acceptable end-products for consumers.

Uticaj skladištene heljde na parametre boje pilećih kobasica tipa emulzije

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Rezime

Standardna formulacija pileće kobasice emulzionog tipa je modifikovana dodavanjem heljdinog brašna skladištenog 0, 3, 6 i 9 meseci kako bi se dobio obogaćen proizvod i pratio efekat promene instrumentalne boje. Kontrola (CON) i četiri kombinovana frankfurter-heljda proizvoda sa brašnom skladištenim na određeni vremenski period (FB0, FB3, FB6 i FB9) su analizirani na svojstva instrumentalne boje (L*, a*, b*, *C**, *h*, *S*, ΔE). Rezultati su statistički obrađeni testovima jednofaktorska ANOVA ($P<0.05$) i Tukey-jev HSD post-hoc ($P<0.05$). Dodato heljdino brašno neznatno utiče na L*, a značajno na a* i b*, sa najvišim vrednostima u FB0 (sličan CON), koji je bio svetliji, crveniji i žući od ostalih kombinovanih proizvoda. Vrednost *C** se značajno smanjila ($P<0.05$), dok se vrednost *h* značajno povećala ($P<0.05$) što je uzrokovalo nižu hromatsku čistoću i više žute nijanse kod FB9 (20.65, 45.40). Značajno smanjenje *S* vrednosti rezultiralo je najvećim slabljenjem u ukupnoj percepciji boje kod FB6 i FB9. Primećen je trend povećanja ΔE, što ukazuje na diferencijaciju kobasica u pogledu uticaja na ukupnu boju ($\Delta E \leq 5{,}08$). Rezultati ukazuju da su pileće kobasice emulzionog tipa obogaćene skladištenim heljdinim brašnom tehnološki opravdane i po boji senzorno prihvatljive kao gotovi proizvodi za potrošače.

Ključne reči: instrumentalna boja, heljda, pileća kobasica, obogaćeni proizvod

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Conflict of interest
The authors declare no conflict of interest.

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CORRELATION OF HEAVY METALS IN MILK AND DRINKING WATER

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Abstract: This study investigates the potential correlation between heavy metal concentrations in drinking water and in the milk of dairy cows across three major regions of Vojvodina, Serbia: Srem, Banat, and Bačka. A total of 360 cows from 18 randomly selected dairy farms (six per region) were included in the study. From each farm, milk samples and drinking water samples were collected and analyzed for the presence of lead (Pb), cadmium (Cd), nickel (Ni), chromium (Cr), and arsenic (As). The aim was to determine the extent of transfer of these heavy metals from water into milk, which could pose a risk to both animal health and food safety. Correlation analyses were conducted separately for each region. Significant positive and negative correlations were observed between water and milk concentrations of specific metals, with notable regional differences. In Banat, strong positive correlations were found between Pb in water and Pb in milk ($r = 0.953$), and Ni in water and Ni in milk ($r = 0.968$). In contrast, Bačka and Srem showed more varied patterns, with both positive and negative correlations across the measured parameters. The findings highlight the need for continuous monitoring of water quality on dairy farms and their implications for milk safety in the region.

Key words: cows, heavy metal, water, milk

Introduction

Milk is a fundamental component of the human diet, particularly for children and vulnerable populations, due to its rich nutritional profile (Ballard and Morrow, 2013; Górska-Warsewicz et al., 2019). However, its safety and quality can be significantly influenced by environmental factors, especially contamination by heavy metals (Masindi and Muedi, 2018; Olowoyo et al., 2024; Rabbani et al., 2025). These contaminants can enter the dairy production chain through various pathways, including feed, air, soil, and most notably, drinking water (Khaniki,

2007; Safaei et al., 2021; Shi et al., 2021). Once ingested, heavy metals such as lead (Pb), cadmium (Cd), nickel (Ni), chromium (Cr), and arsenic (As) can accumulate in the tissues and bodily fluids of livestock, including milk, posing serious health risks to consumers (Balali-Mood et al., 2021).

Dairy cows are particularly vulnerable to environmental contaminants due to their high water and feed intake, making the quality of drinking water a critical point of concern (Zhou et al., 2019). Heavy metal exposure not only affects animal health and productivity but may also lead to the transfer of toxic elements into milk, thereby compromising food safety (Davidov et al., 2019). This is especially concerning in regions where industrial or agricultural activities may increase environmental heavy metal levels.

The region of Vojvodina, located in the northern part of Serbia, is known for its extensive agricultural activities and dairy production. It consists of three main geographical and administrative regions—Srem, Banat, and Bačka—each with distinct environmental and agricultural characteristics. Despite the economic importance of dairy farming in this area, limited data are available regarding the potential contamination of milk by heavy metals and the environmental factors influencing this process.

Therefore, the objective of this study was to assess the correlation between heavy metal concentrations in drinking water and their presence in cow's milk in dairy farms across Srem, Banat, and Bačka. By evaluating these relationships, this research aims to provide a better understanding of environmental impacts on milk quality and contribute to developing effective monitoring and mitigation strategies for food safety in the region.

Materials and Methods

The study was conducted in the northern Serbian province of Vojvodina, focusing on three major agricultural regions: Srem, Banat, and Bačka. These regions are known for their significant contributions to Serbia's dairy sector and share similar climatic and soil conditions, though they differ in local agricultural practices. A total of 18 conventional dairy farms were selected for the study, with six farms randomly chosen from each region.

The study included 360 lactating dairy cows of various breeds, including Holstein-Friesian, Simmental, and crossbreeds, reflecting the diversity of breeds commonly used in regional milk production. From each farm, 20 cows were randomly selected for sampling, ensuring broad representation without breed bias.

Milk samples were collected aseptically during routine morning milking sessions. Approximately 50 mL of milk per cow was collected into sterile containers and transported under refrigerated conditions (4°C) to the laboratory for analysis.

Drinking water samples (10 mL) were collected from the main water sources used for cattle on each farm. Samples were collected in acid-washed polyethylene bottles, immediately preserved with nitric acid (pH < 2), and stored at 4°C until analysis.

Both milk and water samples were analyzed for concentrations of five heavy metals: lead (Pb), cadmium (Cd), nickel (Ni), chromium (Cr), and arsenic (As). The analyses were carried out using Atomic Absorption Spectrophotometry (AAS) with a PerkinElmer AAnalyst 400 spectrophotometer (manufactured by PerkinElmer, USA). Element-specific AAS techniques were selected based on the analyte's physicochemical properties and expected concentration ranges. Flame AAS (FAAS) was used for the determination of Ni and Cr, as these elements are typically present in higher concentrations and exhibit good sensitivity with flame atomization. Graphite furnace AAS (GFAAS) was employed for Pb and Cd due to their lower concentrations and the need for increased detection sensitivity. Arsenic (As) was analyzed using hydride generation AAS (HG-AAS), given the element's chemical behavior and the necessity for enhanced sensitivity and selectivity in trace-level detection.

Milk samples were prepared using a standard wet digestion method. Specifically, 5.0 mL of each milk sample was accurately measured into a digestion vessel. To each sample, 5.0 mL of concentrated nitric acid (HNO_3, analytical grade) was added, followed by 2.0 mL of hydrogen peroxide (H_2O_2, 30%). The digestion was performed under controlled heating on a hot plate, beginning with gradual temperature increase up to 120 °C and maintained until the solution became clear, indicating complete organic matrix decomposition. The digested samples were then cooled, diluted with deionized water, and filtered through Whatman No. 42 filter paper into 25 mL volumetric flasks. Final volumes were adjusted with deionized water prior to AAS analysis.

Water samples were filtered through 0.45 µm membrane filters and acidified to pH < 2 with concentrated nitric acid to preserve the metal ions in solution until analysis.

All reagents used were of analytical grade, and all glassware was acid-washed. Quality assurance measures included the use of procedural blanks, duplicate samples, and certified reference materials to validate analytical accuracy and precision.

The detection limits (LODs) of the AAS method for the targeted metals were as follows: Pb – 0.5 µg/L, Cd – 0.1 µg/L, Ni – 1.0 µg/L, Cr – 0.8 µg/L, and As – 0.2 µg/L. These limits were sufficiently low to meet both environmental and food safety assessment requirements.

Method validation was performed using certified reference materials (CRMs) for both water and milk matrices, obtained from recognized suppliers (Sigma-Aldrich). Recovery rates for the target elements ranged from 91% to 105%, indicating acceptable method accuracy. Precision was evaluated through replicate

measurements, with each sample analyzed in duplicate (n = 2). Analytical precision was confirmed by calculating relative standard deviations (RSDs), which were consistently below 5% for all elements.

Additionally, procedural blanks and spiked samples were included in each batch to monitor potential contamination and assess method performance. All instruments were calibrated using multi-element standard solutions, and quality control samples were analyzed periodically throughout the measurement sequence to ensure data reliability.

Pearson correlation coefficients were calculated to evaluate the relationship between heavy metal concentrations in drinking water and corresponding levels in cow's milk. Correlation analyses were performed separately for each region (Srem, Banat, and Bačka) to assess potential geographical variations. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using IBM SPSS Statistics software.

Results and Discussion

The correlation analysis between heavy metal concentrations in drinking water and cow's milk showed regional variability across the three study areas: Banat, Bačka, and Srem. The Pearson correlation coefficients for each metal pair are presented in Tables 1–3.

In Table 1, strong positive correlations were observed between water and milk concentrations of lead (Pb) and nickel (Ni), with values of $r = 0.953$ and $r = 0.968$, respectively. A similarly strong correlation was found for cadmium (Cd), but in the negative direction ($r = –0.953$). A highly significant negative correlation was also observed between water and milk chromium (Cr) levels ($r = –0.377$) and arsenic (As) ($r = –0.394$). In drinking water samples, lead (Pb) concentrations ranged from 3.2 to 15.4 μg/L, cadmium (Cd) from 0.2 to 1.1 μg/L, nickel (Ni) from 4.5 to 20.3 μg/L, chromium (Cr) from 1.8 to 6.7 μg/L, and arsenic (As) from 0.5 to 3.6 μg/L. In cow's milk, the corresponding concentrations ranged as follows: Pb from 2.4 to 11.6 μg/L, Cd from 0.1 to 0.9 μg/L, Ni from 3.1 to 14.8 μg/L, Cr from 1.2 to 5.3 μg/L, and As from 0.3 to 2.1 μg/L.

In Banat, the strong positive correlations observed for Pb and Ni suggest a direct transfer of these metals from water to milk, possibly due to higher environmental contamination (Zhou et al., 2019) or greater bioavailability in this region. The strong negative correlation for Cd may reflect complex metabolic interactions or differences in absorption, storage, or excretion mechanisms. Similar trends were reported in studies by Giri et al. (2020), Li et al., (2021) and Cellone et al. (2023) where regional industrial activities affected groundwater quality and, consequently, milk safety.

Table 1. Correlation between heavy metal concentrations in drinking water and cow's milk (Banat)

Sample		Milk				
		Pb	Cd	Ni	Cr	As
H_2O	Pb	0.953	0.367	0.968	-0.377	-0.394
	Cd	-0.953	-0.401	-0.993	0.263	0.330
	Ni	0.065	-0.265	0.184	-0.217	0.142
	Cr	-0.108	0.082	-0.067	0.594	0.156
	As	-0.350	-0.706	-0.168	0.932	0.315

In Table 2, the correlations were more moderate and mixed. A strong negative correlation was observed between water and milk cadmium (r = –0.930), while arsenic in water showed a strong positive correlation with nickel in milk (r = 0.764) and lead in milk (r = 0.761). However, most other values ranged from weak to moderate correlations, both positive and negative. In drinking water, lead (Pb) concentrations were within the range of 2.7–13.1 μg/L, cadmium (Cd) ranged from 0.1 to 0.8 μg/L, nickel (Ni) from 3.9 to 17.5 μg/L, chromium (Cr) from 2.0 to 6.3 μg/L, and arsenic (As) from 0.6 to 3.2 μg/L. Corresponding concentrations in cow's milk were estimated as follows: Pb from 2.0 to 9.7 μg/L, Cd from 0.1 to 0.7 μg/L, Ni from 2.5 to 12.6 μg/L, Cr from 1.1 to 4.9 μg/L, and As from 0.3 to 1.9 μg/L.

Table 2. Correlation between heavy metal concentrations in drinking water and cow's milk (Bačka)

Sample		Milk				
		Pb	Cd	Ni	Cr	As
H_2O	Pb	-0.095	-0.425	-0.160	-0.364	0.281
	Cd	-0.930	-0.196	-0.845	-0.361	0.746
	Ni	-0.563	0.291	-0.243	-0.039	0.902
	Cr	0.588	-0.525	0.516	-0.441	-0.039
	As	0.761	-0.233	0.764	-0.248	-0.023

The results from Bačka indicate weaker and more variable correlations. The strong negative relationship between Cd in water and milk mirrors findings from Banat, supporting the hypothesis that cadmium bioaccumulation may be inhibited or regulated in dairy cows. However, the strong positive correlation between arsenic in water and certain metals in milk (Pb and Ni) may indicate

synergistic effects or shared absorption pathways (Tchounwou et al., 2012; Chirinos-Peinado et al., 2024; Angon et al., 2024; Preonty et al., 2025).

In Table 3, a strong positive correlation was found between water and milk nickel (r = 0.955), while water and milk chromium showed a strong negative correlation (r = –0.676). Cadmium and chromium in water were negatively correlated with their concentrations in milk (r = –0.666 and r = –0.676, respectively). Notably, no correlations were observed for arsenic in this region, with all values recorded as zero. In water samples, the concentrations were estimated as follows: lead (Pb) ranged from 3.0 to 12.8 μg/L, cadmium (Cd) from 0.2 to 0.9 μg/L, nickel (Ni) from 4.0 to 18.7 μg/L, chromium (Cr) from 1.5 to 6.0 μg/L, and arsenic (As) was consistently below the detection limit. In milk samples, Pb concentrations ranged from 2.1 to 9.2 μg/L, Cd from 0.1 to 0.6 μg/L, Ni from 3.2 to 13.4 μg/L, Cr from 1.0 to 4.5 μg/L, while As remained undetected in all samples.

Table 3. Correlation between heavy metal concentrations in drinking water and cow's milk (Srem)

Sample		Milk-				
		Pb	Cd	Ni	Cr	As
H_2O	Pb	-0.247	0.116	0.955	-0.242	0
	Cd	0.119	-0.666	-0.316	-0.702	0
	Ni	0.230	-0.709	-0.563	-0.221	0
	Cr	-0.646	0.145	-0.175	-0.676	0
	As	0	0	0	0	0

In Srem, the presence of a strong water–milk correlation for Ni, along with a marked negative relationship for Cr and Cd, highlights the influence of local water chemistry and possibly differences in farm management or soil composition. The results in this study are similar to Zhou et al. (2019), Hussain et al. (2024) and Kanwal et al. (2024). The absence of any detectable correlation for As in Srem is notable and may reflect either consistently low environmental levels or limitations in detection sensitivity.

The results of this study demonstrate a measurable relationship between heavy metal concentrations in drinking water and their presence in cow's milk, with notable differences observed between the three regions of Vojvodina. These findings suggest that environmental exposure through water intake may contribute to the accumulation of toxic metals in milk, potentially posing risks to animal and public health.

Overall, the data underline the importance of region-specific monitoring and environmental management practices. The observed variation in correlation

strength and direction across regions may result from differences in geology, industrial pollution, farming practices, and water source quality. Further investigation, including soil and feed analysis, could provide deeper insights into the pathways and mechanisms of metal transfer in dairy systems.

Conclusion

This study provides valuable insights into the relationship between heavy metal concentrations in drinking water and their presence in cow's milk across three key dairy regions of Vojvodina: Srem, Banat, and Bačka. The findings indicate that certain heavy metal, particularly lead (Pb) and nickel (Ni), show significant correlations between water and milk, suggesting a potential transfer route that could impact milk safety. Regional differences in correlation strength and direction highlight the complex interactions between environmental exposure, animal physiology, and local agricultural practices.

The presence of strong correlations in Banat and Srem, in particular, emphasizes the need for regular monitoring of drinking water quality on dairy farms, as well as the implementation of preventative measures to minimize contamination. While the study did not identify uniformly strong correlations for all metals or regions, the data suggest that targeted surveillance and region-specific management strategies are essential for ensuring milk quality and protecting public health.

Further research involving feed, soil, and blood analysis, along with long-term monitoring, would help clarify the mechanisms of metal transfer and accumulation in dairy cattle. These results can serve as a foundation for developing environmental safety guidelines and risk assessment frameworks for the dairy industry in Serbia and similar agricultural regions.

Korelacija teških metala u mleku i vodi za piće

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Rezime

Cilj istraživanja je da se utvrdi korelacija između koncentracija teških metala u vodi za piće i njihovog prisustva u mleku muznih krava na farmama na teritoriji Vojvodine: Srem, Banat i Bačka. U istraživanje je ukupno bilo uključeno 360 krava sa 18 nasumično odabranih farmi (po šest farmi iz svakog regiona). Sa svake farme uzimani su uzorci mleka i uzorci vode za piće, koji su analizirani na prisustvo

olova (Pb), kadmijuma (Cd), nikla (Ni), hroma (Cr) i arsena (As), radi utvrđivanja stepena prenosa ovih teških metala iz vode u mleko, što može predstavljati rizik kako za zdravlje životinja tako i za bezbednost hrane. Analiza korelacije rađena je posebno za svaki region. Zabeležene su značajne pozitivne i negativne korelacije između koncentracija teških metala u vodi i mleku, sa izraženim regionalnim razlikama. U Banatu su uočene snažne pozitivne korelacije između Pb i Ni u vodi i mleku (r = 0,953 i r = 0,968), dok su u Bačkoj i Sremu rezultati korelacija bili raznovrsniji. Dobijeni rezultati ukazuju na potrebu za kontinuiranim praćenjem kvaliteta vode na farmama muznih krava i sagledavanju posledica koje teški metali mogu imati na bezbednost mleka.

Ključne reči: krave, teški metali, voda, mleko

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

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TRANSFER OF SCIENTIFIC KNOWLEDGE THROUGH TRAINING PROGRAMS FOR ADVISORS IN THE FIELD OF ANIMAL HUSBANDRY

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Abstract: The transfer of scientific knowledge and innovation into practice is one of the key factors in improving the productivity and sustainability of animal husbandry. The goal of this paper is to provide a comprehensive overview of training modules on animal husbandry in the period 2015-2024, aimed at strengthening the competencies of agricultural advisors. Having analysed the data on the animal husbandry trainings (56), the authors were given a complete insight into the dynamics, content, quality and effects of these trainings aimed at improving the knowledge and skills of agricultural advisors. The trainings were grouped by thematic area (10), year (10) and knowledge axes – modernization of knowledge (15 trainings) and knowledge expansion (41 trainings). The effects of the trainings and progress in advisors' knowledge were evaluated by using quantitative and qualitative indicators, established from a survey of the trainees. The results of the research indicate a high level of satisfaction of the trainees, considering that the average rating of all trainings was 4.71 (out of 5.00), the progress in knowledge was 44.56% and the applicability of the trainings was rated 4.58 (out of 5.00), which confirms the quality and relevance of the content and the way the trainings were carried out. In the conclusion of the paper, the authors point out the effect of the trainings of advisors in the field of animal husbandry and a need for some future training programs, while respecting the current trends in agriculture.

Key words: animal husbandry, trainings, knowledge transfer, advisory, survey

Introduction

Over the last decades, agriculture has been facing intense changes, as a reflection of accelerated technological development, digitization, climate change, increasingly strict environmental requirements and the need for sustainable resource management. At the same time, farmers have been under increasing pressure to meet the standards of sustainable production, environmental protection and food safety. Numerous challenges in agriculture require constant adaptation of production practices and rapid adoption of new knowledge, technologies and skills. In this context, knowledge transfer becomes a key factor in the successful development and competitiveness of the modern agricultural sector (Tanasković, 2018; Zecca, 2017; Klerkx and Proctor, 2013). Tolimir et al. (2025) indicate the need to create a new knowledge base according to current challenges in the agriculture and rural development sector. The same authors state that clear line between the period of learning and the period of work is getting blurred, and emphasize how important it is for agricultural experts and farmers to attend trainings after formal education in terms of improvement of agriculture and rural development. Noe et al. (2014) emphasize the contribution of learning based on formal training programs, informal learning and knowledge exchange to the development of human resources. The importance of learning and knowledge transfer between farmers, experts and university teams was also pointed out by Von Munchhausen and Haring (2012). Reddy and Kumar (2020) emphasized that the transfer of modern technologies to farmers requires a well-developed and organized training program, stressing the importance of the choice of training methods.

According to a number of authors, advisory has a very significant role in knowledge transfer (Faure et al., 2012; Đurić, 2020). The role of the agricultural advisory services, as an intermediary between science and practice, is to make the knowledge generated in research institutions available and applicable in the field. In this context, a prerequisite for the effective work of advisors is their continuous professional development and training, in order to respond to complex requirements of modern production and current trends in agriculture (Leeuwis and van den Ban, 2004; Stanković et al., 2015; Tolimir et al., 2025). In the EU countries, the importance of trainings has been recognized and defined by EU regulation no. 1305/2013, which states that in order to increase the quality and efficiency of advice, it is necessary to determine the minimum level of expertise and regular training of advisors. In Serbia, in order to strengthen the competences of agricultural advisors, a continuous training system was established by the Ministry of Agriculture, Forestry and Water Management, defined by the Decree on Establishing the Medium-Term Program for the Development of Advisory Services in Agriculture for the Period from 2021 to 2025, and at the annual level, by the Decree on Establishing the Annual Program for the Development of

Advisory Services in Agriculture. In the advisory system, the first step is to transfer the knowledge from creators to advisors, through trainings of advisors (training-of-trainers), and the second step is to transfer the knowledge from advisors to farmers, as end users, through individual and group methods of work. Part of the research has been devoted to the importance of evaluating advisory programs and measuring their impact on the knowledge and practice of users (Taye et al., 2013). Measuring the impact of training programs on improving the knowledge of farmers, and consequently, agricultural production, was also the subject of research conducted by Rasanjali et al. (2021), and Mariyono et al. (2022).

The aim of this paper is to analyse the trainings of advisors of Advisory Service of Serbia (central Serbia) on animal husbandry that have been carried out over the last ten years, looking at their scope, thematic focus and effects through quantitative and qualitative indicators. A particular focus was put on the grouping of trainings according to the axes of knowledge - knowledge modernization and the knowledge expansion - as well as on the evaluation of the progress of the trainees through the self-assessment of their knowledge before and after the trainings. The results of this research can contribute to a better understanding of the functionality of the existing system of professional development of advisors and be a basis for further improvement of training content in accordance with the needs of modern agricultural practice.

Material and Methods

The research on the trainings of advisors from the Agricultural Advisory and Expert Service of the Republic of Serbia (PSSS), in charge of animal husbandry, during a ten-year period (from 2015 to 2024), included quantitative and qualitative indicators, in order to assess the scope, content and effects of the training activities. This research solely took into account trainings on expert topics, within the axis of knowledge modernization and knowledge expansion, while trainings aimed at strengthening advisory skills within the axis of applied knowledge were not the subject of this analysis.

Quantitative data were collected on the basis of the annual realization of trainings, where the number of trainings and the number of trainees per training were recorded. The trainings were systematized by year, thematic area and axis of knowledge: the axis of knowledge modernization - innovating knowledge on new and modern production technologies, and the axis of knowledge expansion - in areas that had not been covered by regular schooling.

Qualitative indicators were determined through a survey of trainees, with an average of 30 trainees per training session and a total of 1,680 recorded attendances during the examined period. Trainees answered each question, rating them from 1 (lowest) to 5 (highest). After each training, the trainees filled out a

questionnaire containing the following questions: 1) How do you rate the lectures? How do you rate the training materials? 2) How do you rate the possibility to take part in the discussion? (Indicator of training interactivity); 3) How do you rate the training as a whole? 4) How do you rate the applicability of the acquired knowledge? Progress in knowledge was measured with self-assessment tests; before the training, the trainees answered the question “How do you rate your knowledge before the training“, and after the training “How do you rate your knowledge after the training“. Progress in knowledge was expressed as a percentage, and calculated by comparing the initial and final assessment of knowledge.

Standard methods of analysis in the Microsoft Excel were used for data processing.

Results and Discussion

Table 1 shows the results on the quantitative indicators of the trainings - the number of modules by thematic area/year and the number of trainees at the training on a particular thematic area.

Based on the data on the training scope and distribution, it can be stated that there is a continuity in the realization of trainings over the ten-year period, with a total of 56 trainings, and 1,680 trainees. The average number of trainees per training was about 30, which corresponds to the number of PSSS advisors on animal husbandry, indicating that the training includes the entire PSSS staff, thus achieving the main goal - improving the knowledge and competence of all relevant actors. Analysing the number of trainings by thematic area, it can be noted that the focus was on modern production technologies, production economics and the use of funds.

The dynamics of training activities, i.e. the continuity in the implementation of the modules, confirms the strategic approach to the professional development of advisors, which aligns with recommendations that emphasize the importance of permanent education to the improvement of advisory services (Stanković et al., 2015). The knowledge that advisors acquired through permanent education can be considered a basis for providing an effective service to farmers, whether advisors apply traditional models of knowledge transfer (linear, “top down" approach) or use a participatory approach, which implies the interaction between advisors and farmers, i.e. active participation of farmers in the decision-making process, which is increasingly relevant in modern advisory (Prajapati et al., 2025; Vidyawati et al., 2025). Zarokosta and Koutsouris (2024) emphasised the importance of strengthening the capacity of advisors and researchers to support interactive innovation, through mastering some methodological tools that encourage active participation and strengthen innovative networks to integrate actors in a more effective way. Tolimir et al. (2025) pointed out the importance of

trainings in strengthening the capacities of Serbian advisors, given the new concept of AKIS, according to which the role of advisors has changed to being "intermediaries in innovation", which results in the need for a new knowledge, that is, new competences of advisors.

Table 1. Number of training modules and trainees by thematic area and year - quantitative indicators of trainings in the 2015-2024 period

Quantitative indicators of the trainings											
Number of training modules and trainees by thematic area											
Thematic areas	Modern production technologies	Breeding	Organic production	Animal welfare	Diversification of production	Adaptation to climate change	Digitization in production	Rural development	Production economics	Use of funds	Total
Number of modules	9	3	2	4	4	2	4	4	10	14	56
Number of trainees	300	83	42	135	149	75	158	66	295	377	1,680
Dynamics of training modules by year, in 2015-2024 period											
Year	1	2	3	4	5	6	7	8	9	10	10
Number of modules	4	5	5	5	6	7	6	6	7	5	56

Analysing the thematic areas of the modules, one can ascertain their compliance with the challenges in modern animal husbandry, as well as with the current needs of farmers. In order to have some better insight into the subject, the modules were systematized into ten areas, although in a certain way the modules on breeding (genomics), organic production, animal welfare, diversification of production, adaptation to climate change can be grouped in a module on modern technologies (a total of 26 modules), which indicates that, in a broader context, the largest number of the modules belonged to the field of production technology. Considering the role of advisors in rural development, part of the modules were dedicated to this topic. Essentially, all the modules were in the function of the competitiveness of agricultural production, what was one of the criteria for the selection of the modules. Melodillar (2021) pointed out the importance of trainings on production technologies, emphasizing their connection with economically relevant effects, which could also be associated with the applicability of trainings.

Table 2 shows the data on the evaluation of the quality of the lectures, the relevance of the training materials and the degree of training interactivity.

Table 2. Evaluation of the lectures, training materials and opportunities for participation in the discussion - indicators of the training quality in the 2015-2024 period

Qualitative indicators of the trainings – ratings of the lectures, materials, opportunities for discussion, and the trainings as a whole				
	Training rating			
Areas / Knowledge axis	Lecture	Training materials	Discussion	Training as a whole
Production technology	4.76	4.81	4.84	4.80
Breeding	4.80	4.81	4.57	4.76
Organic production	4.58	4.71	4.83	4.76
Animal welfare	4.72	4.78	4.78	4.76
Diversification	4.76	4.83	4.89	4.76
Adaptation to climate change	4.77	4.80	4.89	4.85
Digitization in production	4.70	4.73	4.76	4.77
Rural development	4.84	4.87	4.86	4.71
Production economics	4.63	4.64	4.77	4.80
Use of funds	4.66	4.68	4.80	4.62
Average	4.72	4.77	4.80	4.62

Qualitative evaluation (Table 2) shows high average ratings for the training as a whole (4.75/5.00) and for interactivity (4.80/5.00). The ratings of the quality of lectures and training materials confirm that the topics reflected the real needs of advisors and trends in animal husbandry. The ratings were high in all aspects, indicating a high quality of both the content and the way of presentation, as well as the dynamics of the lectures. After analysing the ratings by thematic area, it can be seen that trainings on professional topics (in animal husbandry) were rated somewhat better, which can be explained by the direct correlation of such topics with advisors' primary professional interests and daily practice, in contrast with trainings on agro-economics that is not their vocation in a narrow sense.

Particularly important was a high rating of the possibility to participate in the discussion, which indicates interactivity as one of the key qualitative aspects of the training process. Interactivity, i.e. the possibility of two-way communication between the lecturer and the trainees, significantly contributes to the quality of learning, because it enables a better understanding of a complex content and the active involvement of all participants (Illeris, 2018). Moreover, high ratings of the interactivity of the trainings indicates that the trainings were carried out following modern approaches to adult education and professional development, which emphasize the importance of participatory learning methods.

In the context of advisory work, the quality of the trainings is particularly important because it directly affects the transfer of knowledge to end users - farmers. In this sense, these results indicate that a potential for multiplying effects

through the practical application of acquired knowledge has been fulfilled through the trainings of advisors.

Table 3 shows the results of trainees' self-assessment of their knowledge before and after the trainings, as well as the assessment of the applicability of acquired knowledge in their advisory work.

Table 3. Knowledge progress and applicability – impact of the trainings

Rating knowledge progress and the applicability of the acquired knowledge		
Areas / Knowledge axis	Knowledge progress according to self-assesment, %	Knowledge applicability
Thematic areas		
Production technology	35.66	4.85
Breeding	50.21	4.35
Organic production	38.12	5.00
Animal welfare	40.49	4.57
Diversification	34.72	4.85
Adaptation to climate change	34.70	4.79
Digitization in production	58.34	4.26
Rural development	52.81	4.48
Production economics	46.02	4.41
Use of funds	42.66	4.57
Average	43.37	4.61
Knowledge axes		
Knowledge modernisation (15 trainings)	36.50	4.73
Knowledge expansion (41 trainings)	45.68	4.52
Average	41.09	4.63

Progress in knowledge is one of the key indicators of the success of the training program, especially in the context of professional trainings for adults, and it amounted to 43.37% for all implemented modules. The obtained results for knowledge progress indicate a well-designed thematic framework and content of the trainings, as well as the implementation of the trainings by using suitable teaching methods and lecturers' effectiveness in knowledge transfer. Looking at the knowledge axes, progress in knowledge was achieved in the axis of knowledge expansion (45.68%) compared to the axis of knowledge modernization (36.50%). It implies that the trainings were particularly effective in areas novel to the majority of the trainees, where there was a large gap between the existing knowledge and innovation. The greatest progress was noted for the following areas: digitization (58%), rural development (52%) and genomic breeding (50%), which are the areas the trainees did not cover through their regular schooling, so such areas were new to them. The complexity of knowledge acquisition is confirmed by research on the

assessment of the adoption of technologies, which according to Ruzzante et al. (2021) depends on different categories, such as age, education, gender, household size, farming experience, land size, land fertility, ownership of land and animals, access to extension services, membership of an organization and other factors.

Apart from the increase in knowledge, the trainees rated very highly the applicability of the acquired knowledge (4.63/5.00) in their daily work, which indicates that the training approach was pragmatic, goal-oriented and aimed at solving specific practical problems. The achieved result is particularly important in the context of advisory and professional services in agriculture, since knowledge from the trainings must be quickly and efficiently transferred to end users, i.e. farmers. The applicability of knowledge is very important - if the knowledge is not directly applied in practice, it is considered to be passive, whereas a high level of applicability indicates that the trainings were aligned with the real challenges and needs of the users. The transfer of knowledge into practice is one of the most important indicators of the success of trainings, whereby Lamm et al. (2017) particularly focused on knowledge management in terms of its creation, transfer and integration of knowledge to be available and usable by certain stakeholders. Research on the transfer of knowledge in the post-training period and systematic monitoring of the application of what has been learned in daily work would make a special contribution to the evaluation of the entire training program in the relationship between knowledge creators, advisors and end users.

Conclusion

Based on the results of the research, which aimed to provide a comprehensive overview of training modules for strengthening the competencies of agricultural advisors of the Advisory Service of central Serbia, for animal husbandry in the ten-year period (2015-2024), it can be concluded that the realized trainings had continuity, were properly structured, thematically relevant and methodologically well implemented, having a significant potential to improve advisors' knowledge and practice in the sector of animal husbandry and rural development.

Qualitative evaluation indicates high satisfaction of the trainees, both in terms of lectures and training interactivity, which indicate that the methods of knowledge transfer were adapted to adult learning and based on the principles of participatory education. Particularly significant are the results on knowledge progress, being highest in areas not previously covered during regular schooling. A high degree of applicability of knowledge indicates the possibilities for direct practical and market use of knowledge acquired through the trainings.

Further directions of research should focus on monitoring long-term effects of the trainings through the advisory system, in terms of evaluating post-training support to farmers, including their economic and social outcomes.

Transfer naučnih znanja kroz programe obuke savetodavaca u oblasti stočarstva

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Rezime

Transfer naučnih znanja i inovacija u praksu jedan je od ključnih faktora povećanja efikasnosti i održivosti stočarske proizvodnje. Cilj rada je da pruži sveobuhvatan pregled edukativnih modula u oblasti stočarstva, usmerenih na jačanje kompetencija poljoprivrednih savetodavca, u periodu od 2015-2024. godine. Analizom podataka o realizovanim edukacijama u oblasti stočarstva (56) dobio se celovit uvid u dinamiku, sadržaj, kvalitet i efekte edukacija koje su imale za cilj unapređenje znanja i veština poljoprivrednih savetodavaca. Edukacije su sistematizovane po tematskim oblastima (10), godinama održavanja (10) i osama znanja - osa osavremenjavanja (15 edukacija) i osa proširenja znanja (41 edukacija). Kroz kvantitativne i kvalitativne pokazatelje utvrđene na osnovu anketnog istraživanja učesnika edukacija evaluiran je doprinos edukacija i napredak u znanju savetodavaca. Rezultati istraživanja ukazuju na visok nivo zadovoljstva učesnika, s obzirom da je prosečna ocena svih realizovanih edukacija 4,71, napredak u znanju 44,56% i ocena za primenljivost edukacija 4,58, što potvrđuje kvalitet i relevantnost sadržaja i načina realizacije edukacija. U zaključku rada ukazano je na doprinos edukacija savetodavaca u oblasti stočarstva i na potrebe za buduće programe obuka, uz uvažavanje aktuelnih tendencija u oblasti poljoprivrede.

Ključne reči: stočarstvo, edukacije, transfer naučnih znanja, savetodavstvo, anketno istraživanje

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

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Correction by the author

The research by Bengouga Khalila, Lahmadi Salwa, Zeguerou Reguia, Maaoui Moufida, Halis Youcef entitled 'LIVESTOCK IN RURAL PIEDMONT REGIONS OF ALGERIA' which was published in July 2019 in the journal Biotechnology in Animal Husbandry 35(2):199-208, DOI: 10.2298/BAH1902199B; contains a typing error, thus the word *swine* has been mistakenly typed four times instead of swan (page 199, line 10; page 202, Table 1 and the second paragraph; page 209, Rezime/Summary, Serbian word for swan – "labudova").

Editorial

Link to the original paper: https://doi.org/10.2298/BAH1902199B

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Journals:

Petrović M.M., Sretenović Lj., Bogdanović V., Perišić P., Aleksić S., Pantelić V., Petrović D. M., Novaković Ž. 2009. Quantitative analysis of genetic improvement of milk production phenotypes in Simmental cows. *Biotechnology in Animal Husbandry*, 25(1-2), 45-51.

Books:

Petrović P.M. 2000. *Genetika i oplemenjivanje ovaca.* Beograd: Naučna knjiga.

Book chapter:

Fitzgerald M. 1994. Neurobiology of Fetal and Neonatal Pain. In: Wall P. and Melzack R. (Eds), *Textbook of Pain* (153-163). London: Churchchill Livingstone.

Scientific Meetings:

Škrbić Z., Lukić M., Bogosavljević-Bošković S., Rakonjac S., Petričević V., Dosković V., Stanojković A. 2015. Importance of farm management in reducing broilers skin lesions. In *Proceedings of the 4th International Congress "New Perspectives and Challenges of Sustainable Livestock Production"*, October 7 – 9, Belgrade, 145-158.

PhD Thesis:

Ružić-Muslić D. 2006. Uticaj različitih izvora proteina u obroku na proizvodne rezultate jagnjadi u tovu. Doktorska disertacija. Univerzitet u Beogradu, Poljoprivredni fakultet.

Caetano A.R. 1999. Comparative mapping of the horse (*Equss caballus*) genome by synteny assignment of type-I genes with a horse-mouse somatic cell hybrid panel. PhD Thesis, University of California, Davis.

Citations with organisations as authors:

European Commission. 2010. Functional Foods. http://ec.europa.eu/research/research-eu. Accessed (date)

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European Union. 1999. Laying down minimum standards for the protection of laying hens. Directive 1999/74/EC. *Official Journal of the European Union*, L 203:53-57.

Law on Animal Welfare. 2009. *Official Gazette of RS*, 41/2009.

Rule book, 2010. Rulebook on conditions..... *Official Gazette of the RS*, 6/10, 57/14, 152/20, 115/23.

Software:

Statistica (Data Analysis Software System). 2006. v.7.1., StatSoft, Inc., USA (www.statsoft.com).

Web Links:

Cureton L. 2022. Carbon Capture İn Livestock Farming**.** Available at https://www.armstrongwatson.co.uk/node/4431. Accessed 20.6.2023.

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Editor's office

15th International Symposium

"Modern Trends in Livestock Production"

29th – 31st October 2025, Belgrade, Republic of Serbia

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