UDC636

Print ISSN 1450-9156
Online ISSN 2217-7140

BIOTECHNOLOGY IN ANIMAL HUSBANDRY

CONTENTS

VOL 42, 1
Founder and publisher
INSTITUTE FOR ANIMAL HUSBANDRY
11080 Belgrade-Zemun
Belgrade 2026

Journal for the Improvement of Animal Husbandry

UDC636

Print ISSN 1450-9156
Online ISSN 2217-7140

BIOTECHNOLOGY IN ANIMAL HUSBANDRY

Belgrade - Zemun 2026

Biotechnology in Animal Husbandry 42, (1), 1- 118 . 2026 ISSN 1450-9156
Publisher: Institute for Animal Husbandry, Belgrade-Zemun UDC 636

Publisher
Institute for Animal Husbandry, Belgrade-Zemun, Serbia

Address of the Editor's office
Institute for Animal Husbandry, Autoput 16, P. Box 23, 11080 Belgrade-Zemun, Republic of Serbia Tel. 381 11 2691 611, 2670 121; Fax 381 11 2670 164;
e-mail: biotechnology.izs@gmail.com; www.istocar.bg.ac.rs

Biotechnology in Animal Husbandry is covered by Agricultural Information Services (AGRIS) - Matica Srpska Library - Referral Center; National Library of Serbia - Repository; University Library "Svetozar Markovic", Belgrade, Serbia; SCIndex repository; EBSCO, USA; DOAJ and European Libraries; SHERPA/ROMEO

Annual subscription: for individuals -500 RSD, for organizations 1200 RSD, - foreign subscriptions 20 EUR. Bank account Institut za stočarstvo, Beograd-Zemun 105-1073-11 Aik banka Niš Filijala Beograd.

Journal is published in two issues annually, circulation 100 copies.

The publication of this journal is sponsored by the Ministry of Education and Science of the Republic of Serbia.
Printed: "Goragraf", Ul. Živka Petrovića 11 Zemun,

Biotechnology in Animal Husbandry 42(1), 1-14, 2026 ISSN 1450-9156
Publisher: Institute for Animal Husbandry, Belgrade-Zemun UDC 637.12'639.111.1
https://doi.org/10.2298/BAH2601001G

DOE MILK AS A SUSTAINABLE HIGH-VALUE DAIRY OPTION FOR SMALL-SCALE FARMS

Vesna Gantner[1], Zvonimir Steiner[1], Čedomir Radović[2], Ranko Gantner[1], Boris Ljubojević[3]

[1] Josip Juraj Strossmayer University of Osijek, Faculty of Agrobiotechnical Sciences Osijek, Osijek, Croatia
[2] Institute for Animal Husbandry, Beograd, Serbia
[3] Croatian forests Ltd., Zagreb, Croatia
Corresponding author: Vesna Gantner, vgantner@fazos.hr
Review paper

Abstract: Global dairy production has undergone significant intensification, which has marginalized small-scale farms unable to compete with conventional dairy cattle systems. Therefore, the aim of this paper was to review the production potential, milk quality characteristics, organization of doe farming systems, and economic feasibility of doe milk production, with a focus on its applicability as a sustainable solution for small-scale farms. Doe milk is characterized by relatively low volume but high nutrient density, elevated protein and fat content, and functional properties, including bioactive peptides and potential hypoallergenic traits. Extensive and semi-extensive farming systems allow low-input management, efficient utilization of marginal pastures, and integration of family labour. Economic sustainability is enhanced through product diversification into cheese, fermented products, and specialty functional or cosmetic applications, compensating for limited milk yield. Despite challenges such as seasonal lactation, low milk production, and limited standardization, doe milk represents a promising alternative for small farms, offering high-value niche products and contributing to rural diversification and sustainable livestock production.

Key words: doe milk, milk quality, small-scale farms, niche dairy products, economic feasibility

Introduction

Global dairy production has undergone profound structural and organizational changes over the past several decades. These changes have been driven primarily by intensification, rapid technological development, genetic progress, and increasing specialization of production systems. As a result, milk yields per animal and per farm have increased substantially, contributing to improved efficiency and global milk supply. However, these developments have also led to a strong concentration of production and a continuous increase in average farm size, particularly in developed dairy regions (FAO, 2020; OECD & FAO, 2023). While large-scale dairy systems have benefited from economies of scale, precision feeding, automation, and advanced herd management, including increasing levels of robotization and digital control, small-scale dairy farms have often struggled to remain competitive. Limited access to capital, rising input costs, labor shortages, and increasing regulatory demands have placed significant pressure on family-owned farms, many of which are unable to invest in modern housing systems, high-performance genetics, or advanced digital technologies (Gorton et al., 2009; Meuwissen et al., 2021; Gantner et al., 2025). Consequently, small dairy farms in many regions face economic instability, reduced profitability and, in extreme cases, abandonment, with negative implications for rural livelihoods, landscape management and regional food security. These developments have renewed broader scientific and societal debates on the role, necessity, and future direction of animal production in the 21st century, particularly in relation to sustainability, food security, and rural development (Gantner et al., 2022).

In response to these challenges, farm diversification and the development of alternative livestock production systems have gained increasing attention as strategies to enhance resilience and sustainability of small farms. Diversification into niche products, value-added processing and alternative animal species has been widely recognized as a viable pathway for maintaining farm income while reducing dependence on conventional commodity markets (van der Ploeg et al., 2019). Within this context, alternative dairy species such as goats, sheep, donkeys, mares and camels have attracted growing scientific and commercial interest due to their ability to efficiently utilize marginal land, adapt to extensive production systems and produce milk with distinctive nutritional and functional properties (Park et al., 2017; Claeys et al., 2014).

In contrast to other alternative dairy species, milk production from deer has been only marginally addressed in the scientific literature. In New Zealand in particular, deer-related research and management have largely focused on non-native populations, their impacts, and wildlife management, as well as venison production and velvet antler harvesting (Latham and Nugent, 2017). Consequently, lactation biology and milk utilization in deer have received comparatively little scientific attention. This is particularly notable given the increasing consumer interest in functional foods, natural products and novel dairy items with high nutritional value and strong product differentiation (Li et al., 2023). Doe milk, although produced in relatively small quantities, is characterized by exceptionally high concentrations of protein, fat and minerals, which confer a high nutritional density and significant potential for value-added processing (Li et al., 2023; Park et al., 2017; Claeys et al., 2014).

From a production perspective, doe milk systems are typically associated with extensive or semi-extensive management, low external inputs and strong integration with natural ecosystems (Park et al., 2017; van der Ploeg et al., 2019). These characteristics align well with the resource base of small farms, especially those located in marginal or less-favored areas where intensive dairy cattle production is not economically viable. When combined with direct marketing, on-farm processing and short supply chains, doe milk production may offer a realistic opportunity for small farms to generate income through specialization rather than volume-based competition (van der Ploeg et al., 2019; Meuwissen et al., 2021).

Therefore, the aim of this paper was to critically review the production potential, milk quality characteristics, organization of doe farming systems, and economic feasibility of doe milk production, with particular emphasis on its possible applicability in small-scale farming systems in Europe, especially in marginal and low-input production areas.

Lactation biology and milk production characteristics in does

Climatic conditions, particularly temperature and precipitation patterns, significantly influence population dynamics and biological performance of cervid species, thereby indirectly shaping reproductive outcomes and physiological processes such as lactation (Gavran et al., 2020). Lactation in does is a biologically evolved process optimized to meet the rapid growth and high metabolic demands of fawns. In free-ranging and managed deer populations, reproductive success and offspring survival are strongly influenced by population density, climatic conditions, and habitat quality, which together shape maternal condition and resource allocation during the reproductive cycle (Gavran and Gantner, 2020). This specialization manifests in a milk profile characterized by elevated concentrations of protein, fat and total solids, which provide dense energetic and nutrient support during early life stages (Park et al., 2017; Li et al., 2023). However, this biological adaptation comes at the expense of overall milk volume, resulting in substantially lower yields per lactation compared with conventional dairy species such as dairy cattle, goats or sheep (Landete-Castillejos et al., 2000). Reported values should nevertheless be interpreted within the specific biological and production contexts from which they were obtained. For example, Landete-Castillejos et al. (2000) investigated captive Iberian red deer (Cervus elaphus hispanicus) kept under controlled experimental conditions, whereas Li et al. (2023) studied milk from farmed red deer in New Zealand collected from a commercial herd managed under pasture-based conditions with supplementary feeding.

A defining feature of doe lactation is its pronounced seasonal pattern. In wild and semi-extensive populations, lactation is closely linked to reproductive cycles and environmental cues, including photoperiodic regulation, ensuring that peak nutrient demands of offspring coincide with periods of higher forage availability (Brown, 1992; Bubenik, 2006; Scott et al., 2008; Strickland et al., 2008). Consequently, the lactation curve of does typically displays a distinct seasonal peak followed by a gradual decline, and the overall length of lactation is constrained by reproductive return intervals. This seasonality limits the feasibility of continuous milk production in the absence of controlled breeding and supplementary feeding strategies.

Quantitative data on daily milk yield in deer species remain relatively scarce, largely due to the logistical challenges of sampling and the limited number of captive production systems. Nevertheless, available studies indicate that daily milk yield in red deer does typically averages below 1 liter per day, which is markedly lower than yields observed in conventional dairy species such as goats or cows (Landete-Castillejos et al., 2000; Li et al., 2023). As shown in Table 1, does produce substantially lower milk volumes than cows or goats; however, the high concentration of protein and fat highlights their suitability for value added dairy products. Yield variability is influenced by species and subspecies genetics, maternal condition, nutrition, management system and milking protocols. For example, does with access to high-quality forage and balanced supplementation during peak lactation can sustain higher yields than those maintained on marginal pastures.

Table 1. Lactation and milk composition parameters of does (red deer) compared with conventional dairy species

Parameter	**Red Deer (Does)**	**Dairy Cow**	**Dairy Goat**	**Notes / Comments**
Lactation length, weeks	24–34[1]	40–44[2]	28–32[2]	Deer lactation is seasonal and limited by reproductive cycles
Peak daily milk yield, L/day	0.8–1.2[1]	20–30[2]	2–4[2]	Lower yield in does compensated by higher solids
Total milk solids, %	24–26[1]	12–13[2]	12–14[2]	Includes protein, fat, lactose
Protein, %	6–10[1]	3.2[2]	3.3[2]	High protein content supports fawn growth and cheese production
Fat, %	6–11[1]	3.5[2]	4.0[2]	Contributes to energy density and processing quality
Lactose, %	3–5[1]	4.8[2]	4.5[2]	Slightly lower in deer milk

[1] Li et al. (2023); Landete-Castillejos et al. (2000)
[2] Park et al. (2017)

Despite low volumetric output, the high solid content of doe milk partially offsets the volume deficit, especially in the context of value-added dairy products such as cheese, fermented milk and high protein concentrates (Li et al., 2023; Maidment et al., 2026). Elevated protein and lipid fractions contribute to desirable technological properties during processing, including curd formation and flavor development, which are critical for specialty dairy production (Haenlein, 2004; Claeys et al., 2014). From a biological standpoint, these compositional attributes are consistent with the evolutionary role of deer milk in supporting rapid early growth rather than sustained high volume yield.

Given these biological constraints, doe milk production is inherently more compatible with small-scale, specialized dairy systems rather than large-scale commercial operations. The limited supply and distinctive composition position doe milk as a premium niche product, with most commercial potential linked to product diversification and direct marketing strategies rather than bulk fluid milk sales. Integration into extensive or semi-extensive management systems, where feeding inputs and infrastructure costs are lower, further enhances the economic logic of doe dairy enterprises for small or marginal farms (van der Ploeg et al., 2019; Meuwissen et al., 2021).

The lactational biology of does reflects an evolutionary strategy prioritizing nutrient density over volume. While these limits continuous high-volume milk supply, it simultaneously creates opportunities for specialized dairy products with high nutritional and economic value when integrated into appropriate production and marketing systems.

Milk quality and nutritional characteristics

Doe milk possesses a unique chemical composition that distinguishes it from conventional dairy species and underpins its potential as a high-value niche product. Its high total solids content, including elevated levels of protein, fat, and minerals, provides a dense source of energy and nutrients suitable for both neonatal growth and human consumption (Li et al., 2023; Maidment et al., 2026). Compared with cow and goat milk, doe milk exhibits a distinct casein profile and higher proportions of whey proteins, which can influence digestibility, coagulation behavior and textural properties of processed dairy products (Haenlein, 2004; Park et al., 2017). The mineral fraction of doe milk is particularly notable, with calcium, phosphorus, and magnesium concentrations surpassing those of most conventional dairy species, offering additional nutritional advantages (Claeys et al., 2014; Li et al., 2023). From a functional standpoint, doe milk contains bioactive components with potential health-promoting effects. These include bioactive peptides generated during digestion, medium-chain fatty acids with antimicrobial and metabolic benefits, and immunoglobulins that may support gut health (Haenlein, 2004; Li et al., 2023). Furthermore, differences in protein composition between deer milk and cow milk, particularly in the casein and whey protein fractions, suggest that deer milk may exhibit distinct allergenic properties. However, evidence regarding reduced allergenicity remains limited, and further studies are required to evaluate the potential hypoallergenic characteristics of deer milk in comparison with conventional dairy species (Claeys et al., 2014; Li et al., 2023). Such attributes render doe milk particularly attractive for functional foods and specialized dairy products targeted at health-conscious consumers. Table 2 summarizes the chemical and functional properties of doe milk compared to cow and goat milk, highlighting its high protein, fat, mineral content, and functional potential for niche dairy products.

Table 2. Chemical composition and functional properties of doe milk compared with cow and goat milk

Parameter / Property	Red Deer (Doe)	Dairy Cow	Dairy Goat	Notes / Comments
Calcium, mg/100 g	298[1]	120–130[2]	100–110[2]	Supports bone health; beneficial for cheese texture
Phosphorus, mg/100 g	190–210[1]	90–100[2]	80–95[2]	Important for metabolic and structural functions
Bioactive peptides	Present[1]	Moderate[2]	Moderate[2]	Contribute to antimicrobial, antioxidant, and gut health effects
Immunoglobulins	High[1]	Low[2]	Low[2]	Supports neonatal immunity; potential functional food applications
Hypoallergenic potential	Moderate[1]	Low[2]	Low[2]	Lower β-lactoglobulin; may reduce allergenicity
Recommended product use	Cheese, fermented milk, value-added dairy	Fluid milk, cheese, butter	Cheese, yogurt	Doe milk suited for niche and functional products

[1] Li et al. (2023); Maidment et al. (2026)
[2] Park et al. (2017); Haenlein (2004); Claeys et al. (2014)

The organoleptic qualities of doe milk also contribute to its value. Flavor and aroma profiles, while influenced by diet and lactation stage, are generally mild and pleasant, making the milk suitable for cheese, fermented products, and even direct consumption in markets seeking novel or gourmet dairy experiences (Li et al., 2023; Maidment et al., 2026). Its high solids content not only improves processing yields but also enhances the texture and creaminess of derived products, which can increase consumer acceptability and marketability.

Taken together, the chemical and functional characteristics of doe milk provide strong justification for its development as a niche dairy product. Its high nutrient density, bioactive potential, and favorable processing properties align well with current consumer trends favoring functional, natural, and high-quality foods. Small-scale farms can leverage these qualities to produce value-added products that command premium prices, creating an economically viable alternative to conventional dairy production systems.

Organization of doe farming systems

Doe farming systems are typically based on extensive or semi-extensive management, relying on natural pastures and marginal land resources that are often unsuitable for intensive dairy cattle production. Such systems are characterized by low external inputs and strong integration with local environmental conditions, which aligns well with agro-ecological principles and sustainability-oriented small-farm strategies (Landete-Castillejos et al., 2000; Park et al., 2017; van der Ploeg et al., 2019). Does are highly adaptable animals, capable of thriving across a wide range of environmental conditions, with lactation and reproductive performance closely linked to resource availability and habitat characteristics. This ecological flexibility makes them potentially suitable for low-input and small-scale farming systems. Nevertheless, adequate shelter, basic housing conditions, and protection from adverse weather remain essential to ensure animal welfare, reproductive success, and stable milk production (Strickland et al., 2008; Scott, 2008; Park et al., 2017). Animal welfare in farmed red deer also depends on appropriate housing, stocking density, weather protection, feed and water provision, and species-adapted handling systems. Published reviews of European deer farming standards indicate that welfare requirements include minimum pen size, controlled stocking density, access to shelter, continuous provision of fresh water, and handling practices designed to minimize stress and prevent injury (Urošević et al., 2018). Because red deer are a social and stress-sensitive species, prolonged isolation and poorly designed handling facilities may compromise both welfare and production performance (Urošević et al., 2018).

Feeding strategies in doe milk production are low-input yet nutritionally sufficient, emphasizing seasonal pasture availability complemented by strategic supplementation during lactation. Pasture-based systems provide the majority of energy and fiber requirements, while small quantities of concentrate or protein-rich supplements during peak lactation enhance milk yield and support maternal condition (Li et al., 2023; Park et al., 2017). Compared with dairy cattle, feed and housing costs are significantly lower, reducing the financial barrier for small or family-run farms and increasing overall economic feasibility.

Milking does presents specific challenges linked to both anatomical and behavioral traits. Deer are highly sensitive animals, and their management during calving and lactation requires low-density systems and facilities adapted to their behavioral characteristics. In New Zealand high-country systems, breeding hinds are typically set-stocked at low population densities in large paddocks during calving and lactation, whereas more intensive lowland systems operate at much higher stocking densities (Wall et al., 2019). Such differences in management context are important when considering the practical organization and welfare implications of doe milk production. Manual milking or small-scale adapted milking equipment is typically employed, given the relatively small udder size and lower daily milk yield. Careful attention to hygiene is paramount, as the small volume of milk must be maintained at high sanitary standards for processing into premium dairy products such as cheese, fermented milk, and specialty functional foods (Claeys et al., 2014; Maidment et al., 2026). Although labor requirements per liter of milk may be higher than in conventional dairy cattle systems, this is generally acceptable in small-scale or family-operated farms, especially when the focus is on value-added production rather than volume-based sales. Table 3 presents the key aspects of doe farming systems, highlighting low-input feeding, milking strategies, and labor requirements suitable for small-scale operations.

Table 3. Organization and management of doe farming systems (Li et al., 2023; Maidment et al., 2026; Park et al., 2017; Claeys et al., 2014)

Aspect	**Doe management**	**Description / Notes**	**Advantages for Small Farms**
Farming system	Extensive / semi-extensive	Utilizes natural pastures, forest edges, marginal land	Low infrastructure costs; aligns with animal welfare
Housing	Minimal shelter / simple barns	Protects from extreme weather; bedding optional	Low capital investment; easy maintenance
Feeding strategy	Pasture-based + seasonal supplementation	Concentrates or protein-rich feed during peak lactation	Low-input feeding; supports milk production and health
Water provision	Fresh, clean water	Ad libitum access required	Supports welfare and milk yield
Milking method	Manual or small-scale adapted equipment	Milking 1–2 times/day; requires careful handling	Flexible for small volumes; avoids large-scale investment
Hygiene and milk handling	Strict hygiene practices	Sanitization of equipment; proper storage at low temperatures	Ensures high-quality milk for premium products
Labor requirements	Moderate to high per unit of milk	Family-operated farms suitable; higher labor per liter compensated by product value	Feasible for small farms; focus on value-added production
Complementary activities	Agro-tourism, educational programs, forest management	Diversification of income; promotes sustainability	Enhances economic resilience; integrates well with niche products

The organization of doe farms can also integrate complementary activities, such as agro-tourism, forest management, and educational programs, which further increase income diversification and farm sustainability. Efficient herd management includes careful breeding planning, lactation monitoring, and health surveillance, all of which are critical for optimizing milk yield and maintaining high-quality milk standards (Meuwissen et al., 2021; Li et al., 2023). By combining low-input feeding, extensive grazing, and attention to welfare, small farms can establish economically viable and environmentally sustainable doe milk production systems.

Economic potential and market opportunities

Doe milk production presents a unique economic model in which profitability is driven more by product value than by production volume. At present, the most clearly documented commercial context for deer milk production is New Zealand, where a limited number of specialized farms have established milking systems based on seasonal production and once-daily milking. In these systems, deer milk is processed into niche, high-value products such as powders, cheeses, desserts, and cosmetic products, although production remains small-scale and highly specialized (Dowd, 2022). Recent research on red deer milk from New Zealand also confirms active interest in the use of deer milk as a novel ingredient in food, nutritional, and cosmetic products (Li et al., 2023). In contrast, European markets for doe milk are still largely underdeveloped, and potential commercialization is currently more likely to depend on product differentiation, short supply chains, direct sales, and integration with complementary farm activities than on standardized large-scale dairy production (van der Ploeg et al., 2019; Meuwissen et al., 2021). Production costs are relatively low, primarily due to minimal infrastructure requirements, reduced feed inputs, and lower veterinary expenses compared with conventional dairy cattle systems (Park et al., 2017; Li et al., 2023). These cost advantages are particularly pronounced in small-scale, family-operated farms, where labor is integrated within routine farm activities, and the emphasis is on value-added production rather than large-scale output. Conversely, the market value of doe milk is high, reflecting its rarity, exceptional nutritional composition, and functional properties. This premium positioning allows small farms to capture disproportionate economic returns per liter of milk or per unit of processed product (Haenlein, 2004; Maidment et al., 2026). Table 4 summarizes the key economic parameters and market opportunities for doe milk production, highlighting low costs, high product value, and the potential for diversified, value-added products suitable for small-scale farms.

Table 4. Economic potential and market opportunities of doe milk production (Park et al., 2017; Li et al., 2023; Haenlein, 2004; Maidment et al., 2026; Claeys et al., 2014; van der Ploeg et al., 2019; Meuwissen et al., 2021; Landete-Castillejos et al., 2000)

Aspect	**Description**	**Implications for Small Farms**
Production costs	Low infrastructure, low feed requirements, reduced veterinary expenses	Minimal initial investment; feasible for family-operated farms
Milk market value	High, due to rarity, nutritional quality, and functional properties	Profitability driven by value per liter rather than volume
Product diversification	Cheese, fermented products, specialty dairy items, functional foods, cosmetics	Increases added value and resilience; reduces dependence on single products
Labor requirements	Moderate to high per liter; acceptable in family-operated systems	Compatible with small-scale farms; higher labor offset by product value
Market channels	Direct marketing, farm shops, agro-tourism, niche markets	Short supply chains enhance margins; opportunity for premium pricing
Limitations	Low milk yield, seasonal production, lack of standardization, regulatory barriers	Requires strategic planning, niche marketing, and adaptation

Economic sustainability in doe milk production is strongly enhanced by product diversification. Beyond direct consumption of fresh milk, processing into cheese, fermented milk products, yogurts, and specialty dairy items significantly increases added value (Li et al., 2023). Innovative processing approaches can also yield concentrated proteins, functional beverages, and other specialty products for niche health-oriented markets. Additionally, non-food applications, such as cosmetics, skincare products, and nutraceuticals, offer further revenue streams, leveraging the unique composition of deer milk (Claeys et al., 2014). By diversifying outputs, small farms can reduce dependency on single products and improve economic resilience.

Doe milk production is particularly well-suited for small-scale farms for several reasons. Initial investment costs are modest, feeding requirements are compatible with pasture-based or semi-extensive systems, and family labor can efficiently manage small herds. Furthermore, integration with rural tourism, farm visits, and direct marketing creates additional income opportunities, while short supply chains allow farmers to capture higher margins by selling directly to consumers (van der Ploeg et al., 2019; Meuwissen et al., 2021). On-farm processing further enhances profitability and strengthens farm resilience against market fluctuations. The broader institutional context also plays an important role in shaping the economic feasibility of small-scale dairy systems. Local government strategies aimed at improving the investment climate and supporting rural entrepreneurship can facilitate the development of value-added agricultural activities in rural areas (Dokić et al., 2020).

Despite these advantages, several challenges and limitations must be considered. Doe milk yield remains low and highly seasonal, with production peaks aligned with reproductive cycles and environmental conditions (Landete-Castillejos et al., 2000; Li et al., 2023). Lack of standardized milking technologies, processing protocols, and farm management guidelines can hinder scaling and consistency. Additionally, scientific data on long-term production economics, milk composition variability, and animal welfare in commercial deer dairy systems remain limited. Regulatory frameworks governing milk from non-conventional species are often underdeveloped or inconsistent, potentially restricting market access in some regions (Claeys et al., 2014; Park et al., 2017). Overcoming these constraints requires targeted research, technology adaptation, and supportive policy measures to fully realize the economic potential of doe milk production.

Conclusion

Doe milk production offers a viable alternative to conventional dairy systems for small-scale farms, combining low-input management with high-value niche products. Despite low and seasonal yields, doe milk's high nutrient density, distinct protein and fat profiles, and potential functional properties make it particularly suitable for specialty dairy products such as cheese, fermented foods, and functional or cosmetic applications. Extensive and semi-extensive farming systems allow efficient use of marginal lands while supporting animal welfare and family labor integration. Economic sustainability relies on product diversification, short supply chains, and value-added processing.

There are still several challenges, including limited yield, seasonal constraints, lack of standardized technologies, and regulatory barriers. Addressing these through research, optimized management, and supportive policies could expand the feasibility and profitability of doe milk production. Finally, doe milk could represent a sustainable, high-value strategy for small farms, contributing to rural diversification and the development of niche dairy markets.

Mleko košute kao održiva i visokovredna mlečna opcija za male farme

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

Rezime

Globalna proizvodnja mleka doživela je značajnu intenzifikaciju, što je marginalizovalo male farme koje nisu u mogućnosti da se takmiče sa konvencionalnim sistemima uzgoja krava. Cilj ovog rada bio je da se izvrši pregled potencijala proizvodnje, karakteristika kvaliteta mleka, organizacije sistema uzgoja košuta i ekonomske održivosti proizvodnje mleka košute, sa fokusom na primenu kao održivo rešenje za male farme. Mleko košute karakteriše relativno nizak prinos, ali visok sadržaj hranljivih materija, povišen sadržaj proteina i masti, kao i funkcionalna svojstva, uključujući bioaktivne peptide i potencijalne hipoalergene karakteristike. Ekstenzivni i poluekstenzivni sistemi uzgoja omogućavaju upravljanje sa niskim ulazima, efikasno korišćenje marginalnih pašnjaka i integraciju porodičnog rada. Ekonomska održivost se dodatno poboljšava kroz diverzifikaciju proizvoda, uključujući sireve, fermentisane proizvode i specijalne funkcionalne ili kozmetičke proizvode, čime se kompenzuje ograničen prinos mleka. Uprkos izazovima kao što su sezonska laktacija, nizak prinos i ograničena standardizacija, mleko košute predstavlja perspektivnu alternativu za male farme, nudeći visoko vredne nišne proizvode i doprinoseći ruralnoj diverzifikaciji i održivoj stočarskoj proizvodnji.

Ključne reči: mleko košute, srna, kvalitet mleka, male farme, nišni mlečni proizvodi, ekonomska održivost

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

References

Brown R.D. 1992. The biology of deer. Springer-Verlag, New York.

Bubenik A.B. 2006. Seasonality of reproduction in deer: endocrine control and adaptation. *Animal Reproduction Science*, 93(3), 165–182.

Claeys W.L., Verraes C., Cardoen S., De Block J., Huyghebaert A., Raes K., Dewettinck K., Herman L. 2014. Consumption of raw or heated milk from different species: An evaluation of the nutritional and health benefits. *Food Control*, 42, 188–201.

Dokić D., Gregić M., Gavran M., Marković B., Gantner V. 2020. The role of local government in strategic planning and stimulating investments in agriculture sector. *GEA (Geo Eco-Eco Agro) International Conference, Book of Proceedings*, 348–355.

Dowd A.M. 2022. An Investigation of Red Deer Milk in New Zealand and the Implications for Future Production. Lincoln University.

FAO. 2020. Dairy Market Review: Overview of global dairy market developments in 2019. Food and Agriculture Organization of the United Nations, Rome. Available at: https://www.fao.org/3/ca8341en/CA8341EN.pdf (Accessed: 20.03.2026).

Gantner V., Ljubojević B., Kasarda R., Ćosić M., Celik K. 2025. Robotization in animal production. *Proceedings of the 8th International Scientific Conference Village and Agriculture*, 13–22.

Gantner V., Zmaić K., Dokić D., Gregić, M. 2022. Nepotrebnost ili nužnost animalne proizvodnje u 21. stoljeću. *Radovi Zavoda za znanstveni i umjetnički rad u Požegi*, 11, 87–96. https://doi.org/10.21857/m8vqrtge09

Gavran M., Gantner V. 2020. Population trends of the most common large game in the hunting area in eastern Croatia in 2008–2018. *Agroznanje*, 21(1), 31–40.

Gavran M., Marković B., Dokić D., Gantner V. 2020. The connection between climate conditions and population size of most represented large game in a hunting area in Eastern Croatia in period 2008—2018. *GEA (Geo Eco-Eco Agro) International Conference*, 364–373.

Gorton M., Hubbard C., Hubbard L. 2009. The folly of European Union policy transfer: Why the Common Agricultural Policy (CAP) does not fit Central and Eastern Europe. Regional Studies, 43(10), 1305–1317.

Haenlein G.F.W. 2004. Goat milk in human nutrition. *Small Ruminant Research*, 51(2), 155–163.

Landete-Castillejos T., García A., Molina P., Vergara H., Garde J., Gallego L. 2000. Milk production and composition in captive Iberian red deer (Cervus elaphus hispanicus): Effect of birth date. *Journal of Animal Science*, 78(11), 2771–2777.

Latham A.D.M., Nugent G. 2017. Introduction, impacts, and management of non-native deer and other hunted ungulates in New Zealand. *Deer Research*, 8(1), 12.

Li S., Saharawat A., Ye A., Dave A., Singh H. 2023. Characteristics of red deer (Cervus elaphus) milk: Lactational changes in composition and processing impacts on structural and gelation properties. *Foods*, 12(7), 1517.

Maidment C.A., Gathercole J., Haines S., Li S., Ye A. 2026. Comparative analysis of red deer milk proteins throughout lactation using quantitative proteomics. *Journal of Dairy Science*, 109(3), 2165-2176. https://doi.org/10.3168/jds.2025-27225

Meuwissen M.P.M., Feindt P.H., Spiegel A., Termeer C.J.A.M., Mathijs E., Mey Y.D., Finger R., Balmann A., Wauters E., Urquhart J., Vigani M., Zawalińska K., Herrera H., Nicholas-Davies P., Hansson H., Paas W. 2021. A framework to assess the resilience of farming systems. *Agricultural Systems*, 176, 102656.

OECD/FAO. 2023. OECD-FAO Agricultural Outlook 2023-2032, OECD Publishing, Paris. https://doi.org/10.1787/08801ab7-en.

Park Y.W., Haenlein G.F.W., Wendorff W.L. 2017. Handbook of milk of non-bovine mammals. 2nd ed. Wiley-Blackwell.

Scott I.C., Asher G.W., Archer J.A., Littlejohn R.P. 2008. The effect of season on milk yield and composition in farmed red deer (Cervus elaphus). *Journal of Animal Science*, 86(4), 857–864.

Strickland B.K., Demarais S., Keyser P.D., Aiken M. 2008. Variation in mass and lactation among cohorts of white-tailed deer (Odocoileus virginianus). *Wildlife Biology*, 14(3), 315–325.

Urošević M.I., Esattore B., Saggiomo L., Ristić Z. A., Stojanac N. 2018. Animal welfare standards in red deer (Cervus elaphus) farming. *Archives of Veterinary Medicine*, 11(2), 11–20. https://doi.org/10.46784/e-avm.v11i2.22

Van der Ploeg J.D., Renting H., Brunori G., Knickel K., Mannion J., Marsden T., de Roest K., Sevilla-Guzmán E., Ventura F. 2019. Rural development: From practices and policies towards theory. *Sociologia Ruralis*, 49(3), 1–18.

Wall A.J., Asher G.W., Netzer M.S., Johnson M.G. H., O’Neill K.T., Littlejohn R.P., Cox N. 2019. Farmed red deer home range, habitat use and daily movement patterns in a Southland, New Zealand, tussock grassland over calving and lactation. *Animal Production Science*, 59(3), 549–563. https://doi.org/10.1071/AN17516

Received 5 February 2026; accepted for publication 24 April 2026

Biotechnology in Animal Husbandry 42(1), 15-33, 2026
Publisher: Institute for Animal Husbandry, Belgrade-Zemun

ISSN 1450-9156
UDC 636.09'636.1
https://doi.org/10.2298/BAH2601015R

LABORATORY DIAGNOSIS AND BIOSECURITY OF SELECTED EQUINE VIRAL INFECTIONS IN BREEDING POPULATIONS

Andrea Radalj[1], Dejan Krnjaić[1], Isidora Prošić[1], Aleksandar Nikšić[1], Ana Vasić[2], Jelena Živak Ćuk[3], Jakov Nišavić[1]

[1] Faculty of Veterinary Medicine, University of Belgrade, Bulevar oslobođenja 18, 11000, Belgrade, Republic of Serbia
[2] Scientific Institute of Veterinary Medicine of Serbia, Janisa Janulisa 14, 11000, Belgrade, Republic of Serbia
[3] Veterinary Service Centre, Ministry of Defense, Crnotravska 17, 11000, Belgrade, Republic of Serbia
Corresponding author: Andrea Radalj, andrea.zoric@vet.bg.ac.rs
Review paper

Abstract: Viral infections represent a persistent health and economic challenge in equine breeding populations because of their impact on reproductive efficiency, respiratory health, neurological disorders, and the overall stability of breeding operations. This review examines selected viral pathogens of relevance in breeding horses, with particular focus on infections that represent diagnostic and biosecurity challenges: equine herpesviruses 1 and 4, equine arteritis virus, equine influenza virus, and West Nile virus as a regionally relevant vector-borne agent. This review further highlights the characteristics of breeding practices that facilitate virus introduction, maintenance, and transmission, including animal movement, seasonal breeding patterns, intensive contact structures, and stress-associated reactivation of latent infections. Furthermore, the diagnostic value of different sample types and sampling strategies is discussed, as well as the benefits and limitations of molecular, virological, and serological methods in the investigation of respiratory, reproductive, and neurological diseases. Interpretation of laboratory findings within an appropriate clinical and epizootiological context is particularly emphasized, as test results alone may be insufficient for accurate assessment of infection status or herd-level risk. Additionally, this review discusses key biosecurity principles for prevention and control and outlines current challenges and future priorities for equine viral disease surveillance and management in Serbia. Effective control of equine viral infections in breeding populations requires the integration of timely laboratory diagnosis, structured surveillance, and consistently applied biosecurity measures.

Key words: equine viral infections, breeding populations, laboratory diagnosis, biosecurity, surveillance

Introduction

Viral infections represent an important challenge to the health, welfare and economic performance of equine breeding populations. Pathogens such as equine herpesviruses 1 and 4 (EHV1 and EHV4), equine influenza virus (EIV), equine arteritis virus (EAV), and West Nile virus (WNV) cause respiratory, reproductive and neurological diseases, often leading to abortion, neonatal death, loss of valuable breeding animals, disruption of breeding activities, and trade restrictions (Patel and Heldens, 2005; Balasuriya, 2014; Lunn et al., 2024; Cavalleri et al., 2022). In many regions, these viruses persist through endemic circulation, repeated introduction via horse movement, or maintenance in latently infected or carrier animals, making them a continuous rather than occasional threat (Lunn et al., 2024). Timely and accurate laboratory diagnosis is essential for limiting their impact in breeding settings since clinical signs of these viral infections may overlap, be mild or nonspecific. However, laboratory confirmation of infection reduces losses only if it is combined with adequate biosecurity measures. Therefore, control is based on minimizing opportunities for virus introduction and onward transmission through quarantine of new arrivals, prompt isolation of suspect cases, strict hygiene, and management practices that reduce stress and commingling of risk groups (Lunn et al., 2024; Pavulraj et al., 2021). Equine viral infections matter not only because of their direct pathogenic effects, but because failures in early recognition and biosecurity allow endemic and emerging viruses to convert manageable cases into abortion storms, widespread respiratory epizootics, and clusters of severe neurological disease, with long-term repercussions for genetic programmes, animal welfare and the economic sustainability of the horse industry. This review evaluates laboratory diagnosis and biosecurity measures for selected equine viral infections in breeding populations, with particular emphasis on practical implications for surveillance and control in Serbia. The paper focuses on infections that illustrate key diagnostic and biosecurity challenges in breeding populations, including respiratory, reproductive, venereal, latent/carrier-associated and regionally relevant vector-borne infections.

Features of equine breeding populations influencing viral transmission

Certain characteristics of equine breeding systems increase the likelihood of virus introduction and spread, directly influencing diagnostic priorities and biosecurity planning. Breeding studs often house large numbers of mares, foals, and stallions in close proximity. In such settings the horses share airspace, while shared equipment and personnel further facilitate fomite transmission. Environmental studies on EHV1 show that viable virus particles can persist up to 48 h on materials common in stables, providing short term environmental reservoirs in housing areas (Saklou et al., 2021). Another study reported a markedly higher herpesvirus positivity rate in dense breeding environments, with horses housed in stables testing positive for at least one EHV more often than animals kept on pasture or housed individually (Radalj et al., 2021a).

In equine breeding populations, EHV4 and EIV spread most efficiently under conditions of close contact, shared stabling or airspace, frequent animal movement and mixing, and management or environmental stressors that increase susceptibility and viral shedding (Pavulraj et al., 2021; Pusterla et al., 2022). For EHV4, outbreaks are further facilitated by seasonal and management-related stress, housing of foals and mares together, high-density accommodation, limited separation of age groups, and reactivation of latent infections, with possible transmission through air, water, and fomites in breeding farms (Pavulraj et al., 2021; Petano-Duque et al., 2025). For EIV, additional risk factors include enclosed or poorly ventilated housing, stud and sales traffic, introduction of newly transported horses, low or outdated vaccination coverage, waning immunity, mixing of vaccinated and unvaccinated animals, lapses in personnel- or fomite-associated biosecurity, younger age, active training, and weather conditions favoring aerosol spread (Pusterla et al., 2022; Branda et al., 2025).

Horse breeding relies on intensive movement of animals and semen, creating extensive contact networks that facilitate virus introduction between premises (Rosanowski et al., 2013). Trade and transport of semen provides an additional reproductive pathway for viral spread, particularly for EAV and some equine herpesviruses shed by asymptomatic stallions (WOAH, 2024). Movement of breeding stock, including apparently healthy animals, is therefore a major risk for EHV1 introduction, and pre-movement testing alone is insufficient without effective quarantine and biosecurity measures (Barbić et al., 2012). In addition, transport, regrouping, weaning, handling, and seasonal changes impose stress that may trigger herpesvirus reactivation and increased shedding, further amplifying transmission within breeding groups (Radalj et al., 2021a). Together, movement, stress, and high-density mixing are key drivers of EHV1 spread and should be explicitly addressed in stud-farm biosecurity plans (Barbić et al., 2012).

Horses are long day seasonal breeders, therefore foaling and breeding are typically concentrated into a few months. On breeding farms this generates large, relatively uniform cohorts of immunologically naive foals and transiently immunocompromised mares. Seasonally synchronized birth pulses thus periodically increase the pool of susceptible, high shedding youngstock (Stasiak et al., 2022).

Modern reproductive management practices can also influence viral transmission. Centralised stallion stations, artificial insemination centres and embryo transfer facilities bring large numbers of mares through a single site, with repeated close contact between animals, equipment and personnel. Failures in hygienic semen collection, processing, storage, or insemination have been implicated in the spread of venereal and potentially venereally associated viral infections, including EAV, through contaminated semen and equipment (WOAH, 2024).

In combination, these structural features of equine breeding systems generate dense, dynamic contact networks, repeated stressors and frequent mixing of naive and carrier animals, all of which strongly favour the maintenance and rapid spread of endemic and emergent equine viruses. Although these factors are most relevant to directly and indirectly transmitted pathogens, breeding farms may also be exposed to vector-borne infections such as WNV in environments that support mosquito abundance (Petrović et al., 2018).

Equine viral infections

Equine herpesvirus 1 (EHV1) causes respiratory disease, late-term abortion, neonatal foal death and equine herpesvirus myeloencephalopathy (EHM), whereas **equine herpesvirus 4 (EHV4)** predominantly causes upper respiratory disease but can occasionally be associated with abortion (WOAH, 2017). Latent infection is typically established early in life and may persist lifelong, with stress, transport and intercurrent disease triggering reactivation and shedding in apparently healthy carriers, an issue repeatedly documented in Serbia and neighbouring countries (Radalj et al., 2018; Radalj et al., 2021b). Despite widespread vaccination in many breeding regions, outbreaks of respiratory disease, abortion and EHM continue to occur, reflecting incomplete protection against infection and shedding and underlining the importance of strict biosecurity and segregation of pregnant mares from young stock (Barbić et al., 2012).

Equine arteritis virus (EAV) is a major concern for breeding operations because of its ability to cause abortion, neonatal death and persistent infection of stallions with venereal shedding (Balasuriya, 2014). Mares and geldings usually eliminate the virus after acute infection, whereas a substantial proportion of infected intact stallions become long-term carriers, maintaining virus in the accessory sex glands and shedding high titres in semen without loss of fertility (Lazić et al., 2017). Because venereal transmission via natural service or artificial insemination can rapidly disseminate EAV through breeding networks, systematic testing of breeding stallions, semen control, vaccination of EAV-negative colts before first breeding, and tailored management of known carrier stallions are central control measures (Lazić et al., 2017; WOAH, 2024).

Equine influenza virus (EIV), almost exclusively subtype H3N8, is a highly contagious respiratory pathogen capable of explosive outbreaks with very high morbidity in naive groups and serious disruption of breeding, training and trade (Branda et al., 2025). Transmission occurs mainly via aerosols and direct contact, while short-range indirect spread via contaminated equipment and possibly wind-borne aerosols have also been implicated (WOAH, 2019). Although mortality is usually low, EIV outbreaks in breeding herds interrupt stallion services, delay mare movements and sales of young stock, and predispose horses to secondary bacterial pneumonia (Branda et al., 2025). Control in breeding populations relies on maintaining up-to-date vaccination schedules using strains recommended at international level, rigorous biosecurity during high-risk movements, and rapid isolation and movement restrictions when cases occur (Branda et al., 2025).

West Nile virus (WNV) is a mosquito-borne flavivirus that has become endemic across much of southern and central Europe, including Serbia and neighbouring Western Balkan countries. Horses are incidental dead-end hosts but can develop neuroinvasive disease with ataxia, paresis, muscle fasciculations and variable mortality (WOAH, 2018; Cavalleri et al., 2022). For breeding herds in Serbia, regionally relevant arboviral risk is primarily associated with WNV, and key mitigation strategies include vaccination where available, integrated mosquito control, and “One Health” surveillance using horses and wildlife as sentinels for early detection of viral circulation (Lupulović et al., 2011; Vasić et al., 2022; Cavalleri et al., 2022).

The main features of these infections concerning transmission, sampling, diagnosis, interpretation, and biosecurity in breeding populations are summarized in Table 1.

Table 1. Equine viral infections: key diagnostic and biosecurity features

Virus	Relevance	Transmission routes	Recommended samples	Laboratory diagnostics	Diagnostic interpretation	Biosecurity
EHV1 / EHV4	Respiratory disease, abortion, neonatal death, EHM (EHV1)	Respiratory secretions, indirectly via fomites; placenta and aborted tissues (major sources of EHV1)	NP* swabs; EDTA blood; placenta and fetal tissues; paired sera	qPCR (method of choice); VI* (additional tool); VN/CFT* paired serology (surveillance)	Latent infection and subclinical shedding; PCR positivity does not always indicate active disease	Quarantine, isolation of suspect horses, segregation of pregnant mares, hygiene, and movement restriction
EAV	Respiratory disease, abortion, neonatal death, persistent carrier stallions	Respiratory secretions; venereal - via semen from PI* stallions; congenital infection (abortion)	NP swabs; EDTA blood; semen; placenta, fetal tissues and fluids	RT-qPCR (method of choice); VN/ELISA serology (diagnosis and surveillance)	Vaccination and natural infection both induce persistent antibodies; serology may not distinguish infection from vaccination	Stallion testing, semen control, pre-breeding vaccination of EAV-negative colts, and management of carrier stallions
EIV	Explosive respiratory outbreaks causing interruption of breeding activities and movement	Aerosol, direct contact, short-range indirect spread via fomites	NP swabs early after onset; nasal or tracheal washings	RT-qPCR (method of choice); VI (additional tool); sequencing (strain characterization)	Shedding is short-lived, and delayed sampling reduces sensitivity	Up-to-date vaccination, case isolation, movement restriction, and hygiene during high-risk movements
WNV	CNS* disease; regional relevance in Serbia and the Western Balkans	Mosquito-borne transmission	Serum (paired sera; IgM); brain and spinal cord	Serology (method of choice), confirmatory VN/PRN*; RT-qPCR (post-mortem)	Flaviviral cross-reactivity and subclinical exposure may complicate serologic interpretation	Vaccination where available, mosquito control, and sentinel surveillance

*Abbreviations: EHM – equine herpesvirus myeloencephalopathy; NP – nasopharyngeal; VI – virus isolation; VN - virus neutralisation test; CFT – complement fixation test; PI – persistently infected; CNS – central nervous system; PRN - plaque reduction neutralisation.

Sample selection and sampling strategy

For acute respiratory diseases (EHV1/4, EIV, EAV), WOAH recommends upper respiratory tract secretions collected as early as possible after onset of fever and nasal discharge, ideally within the first 24–72 h, when viral load is maximal and shedding is short-lived (notably for EHV1 and EIV) (WOAH, 2017, 2019, 2024; Balasuriya, 2014; Balasuriya et al., 2015). Nasopharyngeal swabs generally yield higher virus titres than simple rostral nasal swabs and are preferred for virus isolation and molecular detection of EIV and EHV1/4 (Pusterla et al., 2008; Chambers and Reedy, 2020). Rayon or polyester-tipped swabs should be inserted deep into the ventral meatus or nasopharynx, rotated against mucosa for several seconds and placed in appropriate viral transport medium or sterile tubes, kept chilled and rapidly shipped (WOAH, 2017, 2024, 2019; Radalj et al., 2022a). Recent work shows that non-invasive samples (nostril wipes, muzzle/nares wipes, environmental stall sponges, air samples) have high agreement with nasal swabs and can support surveillance, particularly in subclinical shedders or when repeated sampling of valuable horses is difficult (Pusterla et al., 2025). However, these should be considered second-choice to deep nasal or nasopharyngeal swabs when the goal is definitive diagnosis of an individual horse (Chambers and Reedy, 2020).

For systemic infections (EHV1, EAV, WNV), WOAH protocols emphasize the use of anticoagulated whole blood (EDTA). In the case of EHV1, optimal detection of cell-associated viraemia by qPCR is achieved when whole blood is collected on days 4–10 post-infection (Balasuriya et al., 2015; WOAH, 2017, 2018, 2024). EDTA blood is a critical sample for EHM diagnosis, where respiratory shedding may already have declined (Giessler et al., 2024). For serological investigations, serum samples are used for the detection of antibodies against EHV1/4, EAV, EIV, and WNV, with paired samples collected 2–3 weeks apart. Blood should be collected aseptically, promptly refrigerated, and separated (for serum collection) as soon as possible to avoid nucleic-acid degradation and haemolysis, both major preanalytical errors for PCR and serology (Padalko et al., 2023).

For laboratory diagnosis in cases of abortion, WOAH identifies the aborted fetus (especially lung, liver, thymus), placenta and fetal fluids as recommended samples for EHV1 and EAV diagnosis since these tissues present with viral titres that are usually higher and more persistent than in the dam (WOAH, 2017, 2024; Balasuriya et al., 2015). Experimental and field data show that aborted fetuses and associated membranes are often superior to nasopharyngeal swabs for EHV1 detection and isolation (Emam et al., 2022). Placenta and fetal lung should be collected as soon as possible after expulsion, kept chilled, and portions frozen for molecular testing (WOAH, 2017, 2024). Correct labelling, individual packaging, and rapid transport prevent the effects of autolysis and environmental contamination that are common causes of false-negative or uninterpretable results.

Vaginal and uterine swabs from aborting mares are important additions to fetal and placental samples for EHV1 and EAV detection and are included in sampling schemes according to WOAH (WOAH, 2017, 2024). Vaginal swabs collected immediately after abortion can yield EHV1 when the fetus and placenta are unavailable or compromised (Emam et al., 2022).

For EAV, WOAH recommends semen (raw and extended, including the sperm-rich fraction) as the most appropriate sample to identify carrier stallions and monitor semen for international trade and artificial insemination programmes (WOAH, 2024; Lazić et al., 2017). Although WOAH prioritizes direct semen testing of breeding stallions, genital swabs (preputial and urethral fossa) can be used as an addition in EAV investigations, particularly when semen sampling is constrained (WOAH, 2024).

EHV1 DNA has also been detected in stallion semen in cases of natural infection, supporting a potential venereal transmission route and justifying semen testing during EHV1 abortion or neurologic outbreaks involving breeding stallions (Balasuriya et al., 2015; Lunn et al., 2024).

Virological laboratory methods and their interpretation

Laboratory methods for detecting equine viral infections increasingly rely on PCR-based assays, which enable rapid, sensitive, and specific detection of viral nucleic acids compared with conventional virus isolation in cell culture and serology (Balasuriya et al., 2015; Milić et al., 2018; Radalj et al., 2018). Real-time PCR (qPCR/RT-qPCR) improves on conventional PCR by enabling sensitive and often quantitative detection, thereby supporting viral load assessment and outbreak management (Milić et al., 2018; Krnjaić et al., 2023; Radalj et al., 2025). Nevertheless, proper laboratory diagnosis relies on the selection of the method according to the phase of infection, tissue tropism, and expected duration of viraemia or shedding. PCR-based assays, particularly qPCR and RT-qPCR, are highly valuable for rapid detection of viral nucleic acids in acute respiratory disease, abortion material, blood during defined viraemic windows, semen from suspected EAV carrier stallions, and post-mortem tissues (WOAH, 2017; WOAH, 2018; WOAH, 2019; WOAH, 2024). Therefore, although samples must be collected within the appropriate diagnostic window, their diagnostic value differs among the selected equine viral infections. This limitation is particularly relevant for WNV, where viraemia in horses is typically short and low-level and diagnosis relies mainly on serology (WOAH, 2018; Cavalleri et al., 2022). As for EAV, semen is preferred over blood to identify persistently infected stallions (WOAH, 2024). Respiratory shedding of EIV is short-lived and delayed sampling reduces PCR sensitivity, while in cases of EHV1 latency and intermittent reactivation complicate interpretation of negative or sporadic positive molecular results

(WOAH, 2017; WOAH, 2019). Multiplex PCR protocols allow simultaneous detection of multiple pathogens or differentiation of closely related viruses such as EHV1 and EHV4 (Radalj et al., 2018). Limitations of PCR methods include the need for specialized equipment, strict quality control, and the inability to distinguish viable from non-viable virus (Radalj et al., 2022b; Krnjaić et al., 2023). Virus isolation in cell culture or embryonated eggs remains critical for obtaining live virus for antigenic characterization, vaccine development, and confirmatory diagnosis, but it is laborious, slower, and less sensitive than qPCR, particularly when viral loads are low, often requiring several passages (Radalj et al., 2018; Chambers and Reedy, 2020; Radalj et al., 2022a; WOAH, 2017, 2019, 2024).

Serological assays, including ELISA and virus neutralization tests, are widely used for surveillance and trade certification because they are relatively inexpensive and scalable. However, as these methods detect antibodies rather than active infection, they may yield false-negative results during the window period and must be properly interpreted to reliably distinguish past from current infection (Krnjaić et al., 2023). Serological methods are important in the diagnosis and surveillance of several equine viral infections. For EAV, virus neutralisation is the reference serological method for detecting previous exposure or vaccination-induced antibodies, while semen testing by virus isolation or RT-PCR is required to determine whether a seropositive stallion is a semen shedder and therefore an epizootiological risk for breeding (WOAH, 2024; HBLB, 2026; IFCE, 2026). For WNV, molecular detection in blood is rarely useful in clinically affected horses because viraemia is usually short and low-level. Therefore, diagnosis is primarily based on serology, particularly IgM-capture ELISA for recent infection, with virus neutralisation or plaque-reduction neutralisation tests used for confirmation because flaviviral cross-reactivity may complicate interpretation (WOAH, 2018; Tolnai et al., 2025). For EIV, RT-qPCR is most useful during the early phase of respiratory disease, whereas haemagglutination inhibition remains an important routine serological test for antibody detection, antigenic characterisation, and subtype differentiation, including distinction between H3N8 and H7N7 viruses when isolates are available (WOAH, 2019; Branda et al., 2025).

Genome sequencing, from targeted genes to whole genomes is the basis of molecular epizootiology, and enables strain typing, detection of reassortment or reversion to virulence, and reconstruction of transmission pathways (Radalj et al., 2018; Petrović et al., 2018; Radalj et al., 2021a; Radalj et al., 2021b; Branda et al., 2025). Overall, molecular, virological, serological, and sequencing methods provide complementary information on infection status, virus viability, immune response, and strain evolution (Radalj et al., 2021b; Krnjaić et al., 2023).

Interpretation of laboratory results in breeding herds requires correlating test results with clinical, reproductive, and management data. For example, the detection of viral nucleic acid by PCR does not necessarily indicate active disease or infectivity. Also, low-level or intermittent positive results may reflect residual, non-replicating virus, contamination, or environmental exposure rather than current pathology (Krnjaić et al., 2023). Quantitative PCR (Cq/viral load) and repeated sampling may therefore help distinguish incidental findings from clinically relevant infection (Hussey et al., 2006). Another important factor complicating interpretation is the presence of latently infected horses in the population. Herpesviruses such as EHV1 establish lifelong latency in neural and lymphoid tissues, with periodic subclinical reactivation and shedding, especially under stress (Radalj et al., 2018). Latent infection is not detectable by routine assays, so PCR-negative horses may still be carriers, and sporadic positive PCR results in apparently healthy animals can reflect subclinical shedding rather than primary outbreaks (Radalj et al., 2021a; Radalj et al., 2021b).

Vaccination can substantially complicate laboratory interpretation because it may induce antibodies indistinguishable from those generated by natural infection, reduce but not eliminate viral shedding, and modify the clinical course of infection (Patel and Heldens, 2005). The effect of vaccination on diagnostic interpretation differs between viruses and tests. For EHV1/EHV4, vaccination may reduce clinical disease and virus shedding but does not provide sterilizing immunity. Therefore, vaccinated horses may still become infected, shed virus, or test PCR-positive after exposure, and serology alone has limited value for distinguishing vaccination-induced antibodies from field infection (Lunn et al., 2024). In this context, interpretation should rely on compatible clinical signs, timing of vaccination, paired serology showing a significant rise in antibody titre, and PCR testing of appropriate clinical samples collected during the expected shedding or viraemic period (Balasuriya et al., 2015). For EAV, vaccination induces long-lasting neutralizing antibodies, which may make seropositive stallions difficult to classify unless their vaccination history is documented. Consequently, seropositive stallions with unknown or incomplete vaccination records should be evaluated by semen testing to determine whether they are carriers and potential semen shedders (Balasuriya, 2014; HBLB, 2026). Vaccination against EIV may modify clinical presentation of the disease and reduce virus shedding, but it does not exclude infection (Branda et al., 2025). Haemagglutination inhibition titres are useful for assessing immune status and antigenic relationships, although interpretation requires knowledge of vaccine strain composition, vaccination date, and whether paired sera demonstrate a recent rise in antibody titre (WOAH, 2019). In WNV diagnostics, this problem is intensified by flaviviral cross-reactivity, as prior immunization or exposure may generate broadly reactive antibodies that obscure identification of the true infecting virus unless confirmatory neutralization assays are applied (Tolnai et al., 2025). Therefore, knowledge of vaccine type, timing, and protocol is essential before attributing a positive result to field infection.

Biosecurity measures for prevention and control

Biosecurity in breeding herds combines bio-exclusion (keeping pathogens out) and biocontainment (limiting spread within the herd) and must be adapted to transmission routes of each virus, epizootiology, and clinical behaviour (Weese, 2014; Crew et al., 2023). Quarantine of newly introduced or returning horses is the basis of bio-exclusion. Recommended measures include physical separation for a defined period, dedicated staff and equipment, temperature monitoring, and targeted testing where appropriate (Weese, 2014; Dominguez et al., 2016). Separation and strict isolation of sick or suspect horses, often using a "traffic-light" cohorting system (infected, exposed, unexposed), are critical to reduce within-farm transmission (Crew et al., 2023). Control of horse movement, on and off the farm and between groups reduces opportunities for virus introduction and spread. Compliance with health certification, vaccination requirements, and event biosecurity protocols has been shown to reduce disease incursions following international movements (Dominguez et al., 2016). Routine hygiene and disinfection are core to both bio-exclusion and biocontainment: hand hygiene, use of personal protective equipment, cleaning and disinfection of stables, foaling areas, equipment, vehicles, and transport boxes, as well as manure and waste management (Weese, 2014; Crew et al., 2023). In breeding practices, management of pregnant mares and foaling units is particularly important. This includes stable, well-drained foaling paddocks, minimizing mixing of mares from different sources, use of proper personal protective equipment and barrier precautions for foaling staff, and written protocols for handling placentas, aborted material, and neonates (Taylor et al., 2020). For vector-borne viruses, insect control (stable design, repellents, and manure management) is essential (Peck et al., 2020). Breeding-specific precautions such as semen testing, stallion health monitoring, and arrival protocols for shuttle stallions further reduce venereal or movement-associated viral risks (Balasuriya, 2014; Dominguez et al., 2016). Effective programs include multiple measures rather than relying on a single intervention, and must be adapted to specific pathogens and facility constraints (Weese, 2014; Crew et al., 2023).

Integration of diagnostics into herd health management

Laboratory diagnostics are most valuable in breeding populations when they are integrated into practical herd-level decision-making rather than used as stand-alone tools. In routine surveillance, targeted testing supports early detection of pathogen circulation, monitoring of immune status, and adjustment of control measures according to the risk profile of the stud (WOAH, 2017, 2018, 2019, 2024). During outbreaks, timely sampling and laboratory confirmation help define cases, identify likely sources of infection, and assess the extent of spread, thereby guiding isolation, movement restriction, vaccination, and reinforcement of biosecurity measures (Weese, 2014; Krnjaić et al., 2023). In breeding practice, diagnostic findings also inform management of abortion events, monitoring of breeding stallions, semen control, and decisions on inclusion or exclusion of horses from breeding groups, thereby linking infection risk with reproductive management and animal movement (Allen and Wilsher, 2018). Their practical value therefore depends on rapid interpretation and effective communication between veterinarians, diagnostic laboratories, and farm managers so that laboratory findings are translated into timely and proportionate control measures.

Current challenges and future directions in Serbia

Control programs for infectious diseases in equine breeding populations in Serbia face several constraints. Subclinical infection and latent carriers are a major problem. Horses infected with EAV can remain lifelong or persistently infected and contagious, often without obvious clinical signs (Lazić et al., 2017). High EAV seroprevalence on stud farms in Vojvodina (21.8%) suggests silent circulation driven by shedder stallions in the absence of systematic control (Lazić et al., 2017). Equine herpesviruses 1, 2 and 5 are widely detected in organs of clinically healthy horses, confirming latent or subclinical infections and potential for intermittent shedding (Radalj et al., 2018). These findings indicate that apparently healthy horses may contribute to silent virus maintenance within breeding populations, emphasizing the need for preventive herd-level biosecurity, movement control, quarantine of introduced or returning animals, targeted monitoring of high-risk groups, and strategic EHV1/EHV4 vaccination as part of integrated disease-control programmes. Repeated serological surveys show high WNV seroprevalence in horses in northern Serbia and Belgrade (up to 70.1%), often without reported clinical disease, indicating frequent subclinical infections and complex interpretation of serological status at the individual level (Veljović et al., 2020; Vasić et al., 2022). Cross-reactivity with Usutu virus in neutralization assays further challenges result interpretation (Lupulović et al., 2011). Although molecular and serological tools exist, unequal access and interpretative difficulties limit their impact. Studies comparing commercial and in-house ELISA and immunofluorescence assays (IFA) for WNV highlight discrepancies between tests and the need for confirmatory assays (Vasić et al., 2022).

In Serbia, mandatory equine disease control measures for the infections considered in this review are focused mainly on surveillance and diagnostics rather than routine compulsory immunization. Equine abortion cases are subject to diagnostic investigation that includes EAV and EHV1. Annual active surveillance is required on holdings with horses, and suspicion of infectious disease must be reported without delay, whereas horse vaccination against EHV1, EAV, and EIV is permitted only according to the epizootiological situation. According to the program established by the Veterinary Directorate of the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia, and conducted in accordance with the Rulebook on the Program of Animal Health Protection Measures for 2025, universal compulsory vaccination against WNV in horses is not established, and its control is primarily based on monitoring and disease suspicion (Official Gazette of the Republic of Serbia, No. 29/2025).

Because persistently infected stallions represent the principal reservoir for venereal EAV transmission, Serbia could adopt a structured breeding-control approach similar to those used in the United Kingdom and France, based on mandatory pre-breeding serology of stallions and semen testing of seropositive or high-risk animals to identify shedders. Confirmed semen-shedding stallions should be excluded from unrestricted breeding or used only under controlled conditions, with documented EAV status, virological testing of semen intended for artificial insemination or natural service, and restrictions maintained until shedding ceases or risk is otherwise mitigated (WOAH, 2024; GOV.UK, 2026; HBLB, 2026; IFCE, 2026; RESPE, 2026).

Future priorities in Serbia should include the establishment of harmonised national surveillance and biosecurity standards for equine viral infections relevant to breeding populations, with particular emphasis on stud farms (Lazić et al., 2017; Petrović et al., 2018). Another important objective is the expansion of integrated surveillance models that combine sentinel horses, vectors, and wildlife, as already demonstrated for West Nile virus (Petrović et al., 2018). Greater use of molecular epizootiology, including whole-genome sequencing and phylogenetic analysis, is also needed to trace EAV transmission clusters and support evidence-based movement control measures (Lazić et al., 2017). In addition, clear, diagnostic algorithms and appropriate decision outputs based on Serbian field data should be developed so that laboratory findings can be translated more consistently into preventive herd health and stud farm management programs (Lazić et al., 2017; Vasić et al., 2022).

Conclusions

Equine viral infections remain a major challenge in breeding populations because of their effects on reproductive performance, respiratory health, and herd stability, and effective control depends on the combined value of accurate laboratory diagnosis and consistently applied biosecurity measures. Diagnostic yield is highly dependent on the samples and context in which tests are used, i.e. correct choice of sampling site, appropriate timing in relation to exposure or onset of signs, and careful interpretation against clinical findings and local epizootiology are essential if results are to reflect true infection status rather than background exposure or latent carriage. Sustainable prevention and control programs in breeding populations must therefore be built on structured surveillance, rapid detection of suspect cases or carriers, movement control, isolation of high-risk animals, and routine cooperation between clinicians, microbiologists, and farm managers so that information flows quickly from the laboratory to the stable. Only the integration of timely laboratory diagnosis with targeted biosecurity measures can provide sustainable control of equine viral infections in breeding populations.

Laboratorijska dijagnostika i biosigurnosne mere u kontroli odabranih virusnih infekcija priplodnih konja

Andrea Radalj, Dejan Krnjaić, Isidora Prošić, Aleksandar Nikšić, Ana Vasić, Jelena Živak Ćuk, Jakov Nišavić

Rezime

Virusne infekcije predstavljaju značajan zdravstveni i ekonomski problem u uzgoju konja imajući u vidu njihov uticaj na uspešnost reprodukcije, zdravlje respiratornog sistema, pojavu neuroloških poremećaja, a time i na opštu stabilnost odgajivačkih sistema. U ovom preglednom radu razmatraju se odabrani virusi konja, sa posebnim osvrtom na konjske herpesviruse tip 1 i 4, virus arteritisa konja, virus influence konja i virus Zapadnog Nila kao regionalno značajan vektorski prenosivi patogen. Razmotrene su specifičnosti odgajivačkih sistema koje pogoduju unošenju, održavanju i širenju virusnih infekcija, uključujući promet životinja, sezonsku dinamiku reprodukcije, kontakte između jedinki i reaktivaciju latentnih infekcija u stresnim uslovima. Osim toga, ukazano je na dijagnostički značaj različitih vrsta uzoraka i strategiju uzorkovanja, kao i na prednosti i ograničenja klasičnih virusoloških tehnika, odnosno molekularnih i seroloških metoda. Posebno je naglašen značaj tumačenja laboratorijskih nalaza u odgovarajućem kliničkom i epizootiološkom kontekstu, imajući u vidu da rezultati laboratorijskih ispitivanja sami po sebi nisu uvek dovoljni za pouzdanu procenu statusa životinje ili rizika na nivou zapata. Pored toga, prikazani su osnovni principi biosigurnosti u prevenciji i kontroli ovih infekcija, kao i aktuelni izazovi i prioriteti budućeg unapređenja nadzora i upravljanja virusnim bolestima konja u Republici Srbiji. Efikasna kontrola virusnih infekcija konja zahteva integrisanu primenu pravovremene laboratorijske dijagnostike, programa nadzora i dosledno sprovedenih biosigurnosnih mera.

Ključne reči: virusne infekcije konja, priplodni konji, laboratorijska dijagnostika, biosigurnost, nadzor

Acknowledgement

The study was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (Contract number 451-03-34/2026-03/200143).

Conflict of interest
The authors declare no conflict of interest.

References

Allen W.R., Wilsher S. 2018. Half a century of equine reproduction research and application: A veterinary tour de force. *Equine Veterinary Journal*, 50: 10-21.
Balasuriya U.B. 2014. Equine viral arteritis. *Veterinary Clinics of North America: Equine Practice*, 30(3), 543-560.
Balasuriya U.B., Crossley B.M., Timoney P.J. 2015. A review of traditional and contemporary assays for direct and indirect detection of Equid herpesvirus 1 in clinical samples. *Journal of Veterinary Diagnostic Investigation*, 27(6), 673-687.
Barbić L., Lojkić I., Stevanović V., Bedeković T., Starešina V., Lemo N., Lojkić M., Madić J. 2012. Two outbreaks of neuropathogenic equine herpesvirus type 1 with breed-dependent clinical signs. *Veterinary Record*, 170, 227.
Branda F., Yon D.K., Albanese M., Binetti E., Giovanetti M., Ciccozzi A., Ciccozzi M., Scarpa F., Ceccarelli G. 2025. Equine influenza: Epidemiology, pathogenesis, and strategies for prevention and control. *Viruses*, 17(3), 302.
Cavalleri J.V., Korbacska-Kutasi O., Leblond A., Paillot R., Pusterla N., Steinmann E., Tomlinson J. 2022. European College of Equine Internal Medicine consensus statement on equine flaviviridae infections in Europe. *Journal of Veterinary Internal Medicine*, 36(6), 1858-1871.
Chambers T.M., Reedy S.E. 2020. Equine influenza diagnosis: Sample collection and transport. *Methods in Molecular Biology*, 2123, 361-367.
Crew C.R., Brennan M.L., Ireland J.L. 2023. Implementation of biosecurity on equestrian premises: A narrative overview. *Veterinary Journal*, 292, 105950.
Dominguez M., Münstermann S., de Guindos I., Timoney P. 2016. Equine disease events resulting from international horse movements: Systematic review and lessons learned. *Equine Veterinary Journal*, 48(5), 641-653.
Emam H.S., Abd-Elhafeiz Y.G.M., Abo-Elkhair M.A., Hesham A.M., Bazid A.H.I. 2022. Detection of local isolates of Equine Herpes virus Type 1 associated with abortion and respiratory manifestation in horses in Giza. *Journal of Current Veterinary Research*, 4(2), 80-90.
Giessler K., Goehring L., Jacob S., Davis A., Esser M., Lee Y., Zarski L., Weber P., Hussey G. 2024. Impact of the host immune response on the development of equine herpesvirus myeloencephalopathy in horses. *Journal of General Virology*, 105(5), 001987.

GOV.UK. 2026. Equine viral arteritis: how to spot and report the disease. Available at https://www.gov.uk/guidance/equine-viral-arteritis Accessed 08.05.2026.

Horse Race Betting Levy Board (HBLB). 2026. International Codes of Practice: Equine viral arteritis. Available at https://codes.hblb.org.uk/page/129 Accessed 08.05.2026.

Hussey S.B., Clark R., Lunn K.F., Breathnach C., Soboll G., Whalley J.M., Lunn D.P. 2006. Detection and quantification of equine herpesvirus-1 viremia and nasal shedding by real-time polymerase chain reaction. *Journal of Veterinary Diagnostic Investigation*, 18(4), 335-342.

Institut français du cheval et de l'équitation (IFCE). 2026. Equine viral arteritis (EVA): a contagious disease circulating in France. Available at https://equipedia.ifce.fr/en/equipedia-the-universe-of-the-horse-ifce/health-and-animal-well-being/diseases/other-diseases/equine-viral-arteritis-eva-a-contagious-disease-circulating-in-france Accessed 08.05.2026.

Krnjaić D., Milić N., Nišavić J., Radojičić M., Radalj A. 2023. Priručnik sa praktičnim vežbama iz Mikrobiologije sa imunologijom 1 i Mikrobiologije sa imunologijom 2. Beograd: Naučna KMD.

Lazić S., Lupulović D., Gaudaire D., Petrovic T., Lazić G., Hans A. 2017. Serological evidence of equine arteritis virus infection and phylogenetic analysis of viral isolates in semen of stallions from Serbia. *BMC Veterinary Research*, 13(1), 316.

Lunn D.P., Burgess B.A., Dorman D.C., Goehring L.S., Gross P., Osterrieder K., Pusterla N., Soboll Hussey G. 2024. Updated ACVIM consensus statement on equine herpesvirus-1. *Journal of Veterinary Internal Medicine*, 38(3), 1290-1299.

Lupulovic D., Martín-Acebes M.A., Lazic S., Alonso-Padilla J., Blázquez A.B., Escribano-Romero E., Petrovic T., Saiz J.C. 2011. First serological evidence of West Nile virus activity in horses in Serbia. *Vector-Borne and Zoonotic Diseases*, 11(9), 1303-1305.

Milić N., Radalj A., Nišavić J. 2018. Standard and molecular methods in the diagnostics of infections caused by equine herpesviruses 1 and 4. *Veterinarski Glasnik*, 72(2), 68-79.

Official Gazette of the Republic of Serbia. No. 29/2025. Available at: https://www.vet.minpolj.gov.rs/dokumenta/program-mera-za-2025/ Accessed 27.03.2026.

Padalko E., Colenbie L., Delforge A., Ectors N., Guns J., Imbert R., Jansens H., Pirnay J., Rodenbach M., Van Riet I., Vansteenbrugge A., Verbeken G., Baltes M., Beele H. 2023. Preanalytical variables influencing the interpretation and reporting of biological tests on blood samples of living and deceased donors for human body materials. *Cell and Tissue Banking*, 25, 509-520.

Patel J.R., Heldens J. 2005. Equine herpesviruses 1 (EHV-1) and 4 (EHV-4)-epidemiology, disease and immunoprophylaxis: a brief review. *Veterinary Journal*, 170(1), 14-23.

Pavulraj S., Eschke K., Theisen J., Westhoff S., Reimers G., Andreotti S., Osterrieder N., Azab W. 2021. Equine herpesvirus type 4 (EHV-4) outbreak in Germany: Virological, serological, and molecular investigations. *Pathogens*, 10(7), 810.

Peck D.E., Reeves W.K., Pelzel-McCluskey A.M., Derner J.D., Drolet B., Cohnstaedt L.W., Swanson D., McVey D.S., Rodriguez L.L., Peters D.P.C. 2020. Management strategies for reducing the risk of equines contracting vesicular stomatitis virus (VSV) in the western United States. *Journal of Equine Veterinary Science*, 90, 103026.

Petano-Duque J.M., Urueña-Martinez E., Cabezas-Callejas L.L., Perilla-Amaya J., Rueda-García V., Rondón-Barragán I.S., Lopera-Vásquez R. 2025. Molecular and Serological Investigation of Equine Herpesvirus Type 1 (EHV-1) and Type 4 (EHV-4) in Horses In Ibagué, Tolima. *Veterinary Medicine International*, 1661949.

Petrović T., Šekler M., Petrić D., Lazić S., Debeljak Z., Vidanović D., Ignjatović Ćupina A., Lazić G., Lupulović D., Kolarević M., Plavšić B. 2018. Methodology and results of integrated WNV surveillance programmes in Serbia. *PLoS One*, 13(4), e0195439.

Pusterla N., James K., Barnum S., Bain F., Barnett D. C., Chappell D., Gaughan E., Craig B., Schneider C., Vaala W. 2022. Frequency of Detection and Prevalence Factors Associated with Common Respiratory Pathogens in Equids with Acute Onset of Fever and/or Respiratory Signs (2008–2021). *Pathogens*, 11(7), 759.

Pusterla N., Lawton K., Barnum S., Magdesian K. 2025. Comparison of nose wipes, stall sponges, and air samples with nasal secretions for the molecular detection of equine influenza virus in clinically and subclinically infected horses. *Viruses*, 17, 449.

Pusterla N., Mapes S., Wilson W.D. 2008. Diagnostic sensitivity of nasopharyngeal and nasal swabs for the molecular detection of EHV-1. *Veterinary Record*, 162(16), 520-521.

Radalj A., Milić N., Stevanović O., Nisavić J. 2021a. The detection and phylogenetic analysis of equine herpesviruses 1, 4 and 5 identified in nasal swab samples of asymptomatic horses from Serbia and Bosnia and Herzegovina. *Veterinaria Italiana*, 57(4), 265-274.

Radalj A., Milić N., Stevanović O., Veljović L., Nišavić J. 2021b. Genetic characterization of equine herpesvirus 1 from clinical cases and asymptomatic horses in Serbia and Bosnia and Herzegovina. *Pakistan Veterinary Journal*, 41(4), 567-573.

Radalj A., Nišavić J., Krnjaić D., Milić N., Prošić I. 2022b. PCR dijagnostika nekih vrsta virusa kod divljih životinja. Primena PCR u otkrivanju gena rezistencije bakterija na fluorohinolone. In *Zbornik predavanja XLIII Seminara za inovacije znanja veterinara*, 25.02.2022, Beograd, 169-173.

Radalj A., Nišavić J., Krnjaić D., Valčić M., Jovanović T., Veljović L., Milić N. 2018. Detection and molecular characterization of equine herpesviruses 1, 2, and 5 in horses in the Republic of Serbia. *Acta Veterinaria Brno*, 87(1), 27-34.

Radalj A., Nišavić J., Milić N. 2022a. Detekcija neurovirulentnih sojeva konjskog herpesvirusa 1. In *Zbornik predavanja XLIII Seminara za inovacije znanja veterinara*, 25.02.2022, Beograd, 49-56.

Radalj A., Prošić I., Nikšić A., Benković D., Krnjaić D., Nišavić J., Stanišić Lj. 2025. Preliminarno ispitivanje prisustva uzročnika kontagioznog metritisa u populaciji konja u Srbiji. In *Zbornik predavanja 16. Naučnog simpozijuma "Reprodukcija životinja"*, 16-19. oktobar 2025, Divčibare, 155-162.

Réseau d'épidémio-surveillance en pathologie équine (RESPE). 2026. Artérite virale équine (AVE). Available at https://respe.net/maladie-equine/avortement/arterite-virale-equine-ave/ Accessed 08.05.2026.

Rosanowski S.M., Cogger N., Rogers C.W. 2013. An investigation of the movement patterns and biosecurity practices on Thoroughbred and Standardbred stud farms in New Zealand. *Preventive Veterinary Medicine*, 108(2-3), 178-187.

Saklou N.T., Burgess B.A., Ashton L.V., Morley P.S., Goehring L.S. 2021. Environmental persistence of equid herpesvirus type-1. Equine Veterinary Journal, 53(2), 349-355.

Stasiak K., Dunowska M., Rola J. 2022. Kinetics of the equid herpesvirus 2 and 5 infections among mares and foals from three Polish national studs. *Viruses*, 14(4), 713.

Taylor K., Thomas S., Mendez D., Chicken C., Carrick J., Heller J., Durrheim D. 2020. "Prevention is the biggest success": Barriers and enablers to personal biosecurity in the thoroughbred breeding industry. *Preventive Veterinary Medicine*, 183, 105135.

Tolnai C.H., Forgách P., Marosi A., Fehér O., Paszerbovics B., Tenk M., Wagenhoffer Z., Kutasi O. 2025. Long-term humoral immune response after West Nile virus convalescence in horses in a geographic area of multiple orthoflavivirus co-circulation. *Journal of Veterinary Internal Medicine*, 39(4), jvim70176.

Vasić A., Răileanu C., Körsten C., Vojinović D., Manić M., Urošević A., Nikolić N., Dulović O., Tews B.A., Petrović T., Silaghi C., Valčić M., Gligić A. 2022. West Nile virus in the Republic of Serbia-Diagnostic performance of five serological tests in dog and horse sera. *Transboundary and Emerging Diseases*, 69(5), e2506-e2515.

Veljović Lj., Maksimović-Zorić J., Radosavljević V., Stanojević S., Žutić J., Kureljušić B., Pavlović I., Jezdimirović N., Milićević V. 2020. Seroprevalence of West Nile fever virus in horses in the Belgrade epizootiological area. *Veterinarski Glasnik*, 74(2), 194-201.

Weese J.S. 2014. Infection control and biosecurity in equine disease control. *Equine Veterinary Journal*, 46(6), 654-660.

WORLD ORGANISATION FOR ANIMAL HEALTH (WOAH). 2017. Equine rhinopneumonitis (infection with equid herpesvirus-1 and -4). Available at https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/2.05.09_EQUINE_RHINO.pdf. Accessed 26.03.2026.

WORLD ORGANISATION FOR ANIMAL HEALTH (WOAH). 2018. West Nile fever. Available at https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/2.01.24_WEST_NILE.pdf. Accessed 26.03.2026.

WORLD ORGANISATION FOR ANIMAL HEALTH (WOAH). 2019. Equine influenza (infection with equine influenza virus). Available at https://www.woah.org/fileadmin/Home/fr/Health_standards/tahm/3.06.07_EQ_INF.pdf Accessed 27.03.2026.

WORLD ORGANISATION FOR ANIMAL HEALTH (WOAH). 2024. Equine viral arteritis (infection with equine arteritis virus). Available at https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/3.06.09_EVA.pdf Accessed 26.03.2026.

Received 16 April 2026; accepted for publication 11 May 2026

Biotechnology in Animal Husbandry 42(1), 35-46, 2026 ISSN 1450-9156
Publisher: Institute for Animal Husbandry, Belgrade-Zemun UDC 637.5.035
https://doi.org/10.2298/BAH2601035L

POSSIBILITY OF SALT REDUCTION IN DRY MEAT

Slobodan Lilić, Danijela Vranić, Branka Borović, Ivana Branković Lazić, Jelena Babić Milijašević, Tamara Gerić

Institute of Meat Hygiene and Technology, 11000 Belgrade, Republic of Serbia
Corresponding author: Slobodan Lilić, slobodan.lilic@inmes.rs
Review paper

Abstract: Excessive sodium intake is widely recognized as a major risk factor for essential hypertension and is associated with a range of adverse health outcomes. According to the World Health Organization, non-communicable diseases represent one of the leading causes of premature mortality worldwide. In response, a global target has been established to reduce population sodium intake by up to 30% by 2025. Meat products constitute a significant source of dietary sodium, particularly fermented sausages and dry-cured meat products. In these products, in addition to low processing and storage temperatures, sodium chloride plays a crucial role as a primary hurdle inhibiting microbial growth and preventing spoilage. Consequently, sodium reduction in such products presents a considerable technological and safety challenge. This is primarily due to the absence of thermal processing, which may increase the risk of microbial proliferation and compromise product safety. This paper examines the potential strategies for sodium reduction in dry-cured meat products, with particular emphasis on microbiological safety, as well as the associated chemical and sensory changes.

Key words: salt reduction, dry meat, sodium

Introduction

Salt (sodium chloride) is one of the earliest and most commonly used food preservatives, particularly for meat (Desmond, 2006). It had significant practical importance, as it enabled longer shelf life for food, especially in winter. Salt intake increased to about 5 g per day and continued to rise until the 19th century when achieved 18 g per day (Roberts and Roberts, 2001).

Excessive sodium intake is recognized as a leading cause of essential hypertension (Hunter et al., 2022). Research indicates that high salt intake leads to increased body mass and elevated total sodium levels in the blood, along with expanded extracellular and plasma volume. At the same time, levels of renin, angiotensin, and norepinephrine are reduced (Haddy, 2006).

Moreover, excessive sodium consumption has been linked to a higher risk of heart attack (Perry and Beevers, 1992), left ventricular hypertrophy (Schmieder and Messerli, 2000), sodium retention in extracellular fluids, and, consequently, the occurrence of clinical and idiopathic edema, particularly in women (MacGregor and de Wardener, 1997). It also contributes to decreased vascular elasticity (Avolio et al., 1986), proteinuria (Du Cailar et al., 2002), greater susceptibility to *Helicobacter pylori* infection and the development of gastric cancer (Tsugane et al., 2004), increased calcium excretion and kidney stone formation (Cappuccio et al., 2000), reduced bone density and osteoporosis (Devine et al., 1995), and more severe asthma attacks (Mickleborough et al., 2005).

According to the World Health Organization (WHO, Fact sheed Nº393), non-communicable diseases, including cardiovascular diseases and stroke, are among the leading causes of premature death worldwide. As a result, a global target was set to reduce sodium intake by 30% by the year 2025. Salt reduction has been identified as one of the most cost-effective public health measures, with estimates suggesting that approximately 2.5 million deaths could be prevented annually (Selmer et al., 2000; Murray et al., 2003; Asaria et al., 2007; Joffres et al., 2007; He and MacGregor, 2009).

In the mentioned document, voluntary global targets are listed:

(1) A 25% relative reduction in the overall mortality from cardiovascular diseases, cancer, diabetes, or chronic respiratory diseases

(2) At least 10% relative reduction in the harmful use of alcohol, as appropriate, within the national context

(3) A 10% relative reduction in prevalence of insufficient physical activity

(4) A 30% relative reduction in mean population intake of salt/sodium

(5) A 30% relative reduction in prevalence of current tobacco use in persons aged 15+ years

(6) A 25% relative reduction in the prevalence of raised blood pressure or contain the prevalence of raised blood pressure, according to national circumstances

(7) Halt the rise in diabetes and obesity

(8) At least 50% of eligible people receive drug therapy and counselling (including glycemic control) to prevent heart attacks and strokes (9) an 80% availability of the affordable basic technologies and essential medicines, including generics, required to treat major noncommunicable diseases in both public and private facilities.

In Table 1, the situation in different regions that have adopted a strategy for reducing salt in food is presented.

Table 1. National sodium/salt reduction initiatives by WHO region (Reducing Salt Intake: What's Happening Around the World, 2024)

Africa	1/47 (2%) South Africa with 2 countries (Ethiopia and Nigeria) in the planning phase
Americas	18/49 (37%) countries / territories with a further 6 countries in the planning stage and 24 without an initiative
Eastern Mediterranean	13/22 (59%) countries
Europe & UK	40/53 (75%) countries / territories
South-East Asia	5/11 countries / territories
Western Pacific	19/37 (51%) countries / territories/areas

Salt reduction in meat products

The most common substitute for sodium chloride is potassium chloride (KCl), but when more than 50% substitution is used, saltiness is significantly reduced and bitterness becomes more pronounced (Armenteros et al., 2012; Barat et al., 2012; Barat et al., 2013). The use of potassium salts is often debated due to potential health risks for sensitive groups, such as individuals with type I diabetes, chronic kidney disease, or heart conditions (FSAI, 2005). Findings of many authors suggest that replacement of sodium chloride with potassium chloride is possible up to 50% without significantly affected color, texture, aroma, or taste.

Over the past decade, studies carried out under projects supported by the Ministry of Education, Science and Technological Development (2010–2019) in the field of technological development have produced numerous scientific outputs related to the reduction of salt content in meat products, and these investigations have continued in recent years. These studies included an analysis of the declared salt content on labels of meat products (Gerić et al., 2021) as well as an evaluation of potential strategies for reducing salt in foods (Xiang et al., 2025). Salt reduction in various groups of meat products was achieved both by decreasing the amount of added salt and by partial substitution of sodium chloride with potassium chloride and ammonium chloride. Particular attention was dedicated to meat products that are not treated by high temperature, such as dried meat products (Lilić et al., 2014; 2015; 2016).

However, salt reduction in dry meat is particular challenge, due to these products are made without any thermal treatment. This is the most important for whole dry hams, where salt penetration occurs slowly, and the inner regions, particularly near the femur and femoral artery, and it presents high microbial risks (Munoz-Rosique et al., 2022; Hernandez Correas et al., 2024).

Relation between salt reduction and microbial status in dry meat

Aliño et al. (2009) investigated the partial substitution of sodium chloride with potassium chloride at a level of 70%, assessing chemical and microbial parameters in dry pork after curing and drying. They found that the salt mixture had no significant impact on the counts of aerobic mesophilic bacteria, halotolerant bacteria, or lactic acid bacteria. The predominant microbiota were halotolerant and lactic acid bacteria.

Sodium chloride does not exhibit a direct antimicrobial effect; its inhibitory action on bacterial growth is primarily due to the reduction of water activity (Devlieghere et al., 2009; Taormina, 2010). According to Prändl (1988), the minimum salt concentrations that inhibit microbial growth are as follows: 5% for *Clostridium botulinum* type E and *Pseudomonas fluorescens*, 6% for *Shigella* and *Klebsiella* strains, 8% for *E. coli*, *Salmonella* spp., *Bacillus cereus*, *Clostridium botulinum* type A, and *Clostridium perfringens*, 10% for *Clostridium botulinum* type B and *Vibrio parahaemolyticus*, 15% for *Bacillus subtilis* and members of the *Streptococcaceae* family, 18% for *Staphylococcus aureus*, 25% for *Penicillium* and *Aspergillus* species, 26% for *Halobacterium halobium*, *Bacterium prodigiosum*, and species of *Spirillum*.

Yamanaka et al. (2005) found that sodium chloride selectively promotes the growth of halotolerant and lactic acid bacteria while suppressing coliform bacteria. The use of salt mixtures containing both sodium and potassium chloride had no significant effect on enterobacteria or coliform bacteria.

Before curing, only Gram-negative bacteria were present in the meat (e.g., *Vibrio*, *Acinetobacter*, *Pseudomonas*, and members of *Enterobacteriaceae*), but during the curing process, the number of Gram-positive bacteria (e.g., *Micrococcus*, *Staphylococcus*, *Pediococcus*) increased, while number of Gram-negative bacteria declined. *Staphylococcus* species are particularly tolerant to high concentrations of sodium chloride (more than 10%), more so than *Micrococcus* species, making their presence expected at the end of the curing period (Yamanaka et al., 2005).

Lorenzo et al. (2015) observed that the number of microorganisms in dry pork changes throughout production. The highest total viable count was recorded in samples cured with a 50:50 mix of sodium chloride and potassium chloride. These findings align with those of Raccach and Henninen (1997), who noted that calcium chloride more effectively inhibits the growth of aerobic mesophilic bacteria compared to a sodium–potassium chloride mixture. However, other studies (Aliño et al., 2010; Blesa et al., 2008) found no significant differences in total viable counts.

Dry meat products made with equal parts sodium and potassium chloride, as well as those with 25% potassium chloride, 20% calcium chloride, and 10% magnesium chloride, contained significantly higher counts of halotolerant bacteria compared to those made with other salt mixtures (Lorenzo et al., 2015). These results contradict findings by Aliño et al. (2010), who reported lower halotolerant bacteria counts when sodium chloride content dropped below 50%. On the other hand, Yamanaka et al. (2005) emphasized that sodium chloride selectively enhances the growth of halotolerant and lactic acid bacteria while suppressing coliforms.

The lowest yeast counts were recorded in dry meat products made exclusively with sodium chloride, indicating that yeasts are sensitive to high sodium concentrations (Lorenzo et al., 2015). However, yeast populations increased significantly toward the end of the curing process, contributing to desirable sensory characteristics due to their lipolytic and proteolytic activity (Purriños et al., 2012, 2015).

Some researchers focused specifically on dry-cured ham and emphasized the importance of maintaining low temperatures (around 3°C) during the post-salting period to prevent microbial growth, especially *Clostridium botulinum* (Ventanas and Cava, 2001). The area at greatest risk for microbial spoilage is the innermost part of the ham, near the femoral artery, due to the lower salt content, higher moisture, and elevated water activity (León-Crespo et al., 1997; Barat et al., 2005). Nevertheless, the substitution of sodium chloride with alternative salts did not result in significant changes in microbial counts (Blesa et al., 2008).

Chemical and sensory changes during salt reduction

Gimeno et al. (1998) investigated the effects of a salt mixture containing sodium chloride (10 g/kg), potassium chloride (5.52 g/kg), magnesium chloride (2.35 g/kg), and calcium chloride (4.4 g/kg), and observed a decrease in pH value. Similar results were obtained with another mixture composed of sodium chloride (10 g/kg), potassium chloride (5.5 g/kg), and calcium chloride (7.4 g/kg) (Gimeno et al., 2001). In contrast, Lorenzo et al. (2015) reported no significant changes in pH value in dry meat products produced with various chloride salt mixtures.

Moisture content was significantly higher in dry meat products made with potassium chloride (Lorenzo et al., 2015; Wu et al., 2014). However, Armenteros et al. (2012) found no significant differences in moisture content between products made with sodium chloride only and those using different chloride salts.

Some studies (Rico et al., 1991; Toldrá et al., 1993) have shown that sodium chloride inhibits the activity of cathepsins B and B+L. Replacing 50–70% of sodium chloride with potassium chloride significantly enhances the activity of these enzymes compared to products treated only with sodium chloride. Cathepsin

H activity does not vary significantly during salting, regardless of the type of chloride salt used. The partial replacement of sodium chloride with 50% potassium chloride increases the activity of cathepsins B and B+L, thereby extending proteolysis. Conversely, dipeptidyl peptidase I activity is higher in meat salted only with sodium chloride, while the activity of dipeptidyl peptidases II and IV remains unchanged. Dipeptidyl peptidase III activity, however, decreases significantly as the share of potassium chloride increases.

Methionyl aminopeptidase activity is inhibited at higher levels of potassium chloride (Armenteros et al., 2009). The activity of aminopeptidases in general is influenced by the type of chloride salts used during meat curing. Specifically, methionyl aminopeptidase is more strongly inhibited with increased potassium chloride content, while alanyl aminopeptidase activity shows no consistent trend when comparing mixtures with and without sodium chloride.

Armenteros et al. (2009) also reported that the most notable changes were observed in sarcoplasmic proteins when sodium chloride was replaced with other chloride salts, while no significant differences were found in myofibrillar proteins. Electrophoretic profiles of myofibrillar proteins revealed intensive degradation of the bonds between myosin and actin during dry-cured meat aging (Toldrá et al., 1993). In contrast, sarcoplasmic protein profiles exhibited greater band density in meat treated with chloride salt mixtures compared to those treated with sodium chloride alone.

The use of alternative chloride salts does not significantly affect lipolytic processes in dry-cured meat. The total content of saturated, monounsaturated, and polyunsaturated fatty acids remains largely unchanged (Armenteros et al., 2009), which is consistent with earlier findings (Countron-Gambotti and Gandemer, 1999) where 50% of sodium chloride was replaced with potassium chloride.

While some findings show consistency, others diverge. For example, Aliño et al. (2009) and Armenteros et al. (2009) demonstrated that up to 50% of sodium chloride could be replaced with a mixture of potassium chloride, magnesium chloride, and calcium chloride in dry-cured pork without significantly affecting the sensory properties of the final product. Similarly, Aliño et al. (2010) found that up to 40% replacement was acceptable in dry-cured ham while maintaining comparable physicochemical characteristics.

The presence of potassium chloride in curing mixtures accelerates the curing process by facilitating faster chloride ion diffusion into deeper muscle layers. As a result, target chloride concentrations are reached more quickly, shortening the overall curing time (Aliño et al., 2010). This is consistent with findings from previous studies (Aliño et al., 2009; Blesa et al., 2008), which confirmed that potassium ions diffuse more rapidly into muscle tissue compared to sodium ions. In contrast, the addition of calcium chloride and magnesium chloride slows the curing process due to the higher electronegativity of their cations, which form stronger bonds with polar groups of proteins, thereby impeding diffusion (Xiong and Brekke, 1991).

Armenteros et al. (2012) found no significant differences in moisture content between dry-cured products made with alternative salt mixtures and traditional products. Conversely, Wu et al. (2014) observed that products made with potassium chloride had significantly higher moisture content than those produced with sodium chloride alone.

Conclusion

Dry-cured meat products are highly valued foods known since ancient times. In most countries, traditional household production has been preserved, as well as modern industrial processing under controlled conditions of drying and fermentation. Since these products are not produced under thermal treatment, their production is classified as high-risk, particularly regarding microbiological spoilage. Therefore, the use of larger amounts of salt is necessary, making the reduction of added salt problematic. The most commonly used replacer for sodium chloride is potassium chloride, whose application is limited by its bitter and metallic taste, allowing only partial replacement of table salt. In most cases, replacement of sodium chloride with potassium chloride is possible up to about 40%. In line with the recommendations of the World Health Organization, these products have also become part of the global initiative to reduce salt in food, which, alongside other meat products, represents a lasting challenge for food science.

Mogućnost smanjenja sadržaja soli u suvom mesu

Slobodan Lilić, Danijela Vranić, Branka Borović, Ivana Branković Lazić, Jelena Babić Milijašević, Tamara Gerić

Rezime

Kuhinjska so (natrijum hlorid) je najstariji i najčešći dodatak hrani, posebno mesu. Unos soli u današnje vreme daleko prevazilazi nutritivne potrebe, a prekomeran unos natrijuma označen je kao vodeći uzrok esencijalne hipertenzije i povezan je sa nekoliko zdravstvenih poremećaja. Zato je postavljen cilj na sastanku u Ženevi da se unos natrijuma smanji za 30% do 2025. godine, shodno preporukama Svetske zdravstvene organizacije. Proizvodi od mesa su najčešće osuđeni kao najveći izvor soli, odnosno natrijuma, iako je ostala hrana, naročito ona procesuirana značajan izvor natrijuma. U ovom radu su prikazane mogućnosti smanjivanja sadržaja soli u suvom mesu, što predstavlja veliki izazov za industriju mesa iz više razloga. Iako je antimikrobni efekat soli zasnovan na činjenici da se snižava aktivnost vode, u ovim proizvodima, i da nema direktan bakteriostatski ili baktericidni efekat, osim niskih temperatura to je jedina prepreka kvaru, naročito u prvim fazama proizvodnje kao što su soljenje i salamurenje. Najveći izazov je proizvodnja suvih šunki, odnosno celih butova svinja zbog velike mase, gde je penetracija soli veoma dug proces, kao i postojanje predilekcionih mesta za mikrobiološki kvar kao što su dublje partije mesa blizu kostiju i dela mesa blizu femoralnog kanala i femoralne arterije. Najčešće korišćena mogućnost smanjivanja sadržaja soli u suvom mesu je parcijalna supstitucija natrijum hlorida drugim solim, najčešće hloridnim solima, u prvom redu kalijum hloridom, što je limitirano njegovim negativnim uticajem na senzorske osobine suvog mesa, u prvom redu pojavom gorkog i metalnog ukusa. Najčešće je ova supstitucija moguća do 50%, bez izrazitih i statistički značajnih razlika u senzorskim karakteristikama. U radu su prikazane mikrobiološke, hemijske i senzorske promene u suvom mesu sa smanjenim sadržajem soli tokom proizvodnje. Pored negativnih uticaja, navedenim postupkom se značajno poboljšava odnos natrijuma i kalijuma, shodno preporukama Svetke zdravstvene organizacije.

Ključne reči: redukcija soli, suvo meso, natrijum

Acknowledgement
The results of this research were financed by the Ministry of Science, Technological Development and innovations of the Republic of Serbia, on the basis of the Agreement on the realization and financing of scientific research work of SRO in 2026, No. 451-03-33/2026-03/200050 dated 05.02.2026.

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

References

Aliño M., Grau R., Toldrá F., Blesa E., Pagán M.J., Barat J.M. 2009. Influence of sodium replacement on physicochemical properties of dry-cured loin. *Meat Science*, 83, 423-430.

Aliño M., Grau R., Fuentes A., Barat J.M. 2010. Characterisation of pile salting with sodium replaced mixtures of salts in dry-cured loin manufacture. *Journal of Food Engineering*, 97, 434-439.

Armenteros M., Aristoy M., Barat J., Toldrá F. 2009. Biochemical changes in dry-cured loins salted with partial replacements of NaCl by KCl. *Food Chemistry*, 117, 627-633.

Armenteros M., Aristoy M.C., Barat J.M., Toldrá F. 2012. Biochemical and sensory changes in dry-cured ham salted with partial replacements of NaCl by other chloride salts. *Meat Science*, 90, 361-367.

Asaria P., Chisholm D., Mathers C., Ezzati M., Beaglehole R., 2007. Chronic disease prevention: health effects and financial costs of strategies to reduce salt intake and control tobacco use. *The Lancet*, 370, 2044–2053.

Avolio A.P., Clyde K.M., Beard T.C., Cooke H.M., Ho K.K., O'Rourke M.F. 1986. Improved arterial distensibility in normotensive subjects on a low salt diet. *Arteriosclerosis*, 6, 166-169.

Barat J.M., Grau R., Ibáńez J.B., Fito P. 2005. Post-salting studies in Spanish cured ham manufacturing time reduction by using brine thawing–salting. *Meat Science*, 69, 201–208.

Barat J.M., Pérez-Esteve E., Aristoy M.C., Toldrá F. 2013. Partial replacement of sodium in meat and fish products by using magnesium salts. *Plant and Soil*, 368, 179-188.

Blesa E., Aliño M., Barat J.M., Grau R., Toldrá F., Pagán M.J. 2008. Microbiology and physico-chemical changes of dry-cured ham during the post-salting stage as affected by partial replacement of NaCl by other salts. *Meat Science*, 78, 135-142.

Cappuccio F.P., Kalaitzidis R., Duneclift S., Eastwood J.B. 2000. Unravelling the links between calcium excretion, salt intake, hypertension, kidney stones and bone metabolism. *Journal of Nephrology*, 13, 169–177.

Countron-Gambotti C., Gandemer G. 1999. Lipolysis and oxidation in subcutaneous adipose tissue during dry-cured ham processing. *Food Chemistry*, 64, 95–101.

Desmond E. 2006. Raducing salt: A challenge for the meat industry. *Meat Science*, 74, 188-196.

Devine A., Criddle R.A., Dick I.M., Kerr D.A., Prince R.L. 1995. A longitudinal study of the effect of sodium and calcium intakes on regional bone density in postmenopausal women. *American Journal of Clinical Nutrition*, 62, 740-745.

Du Cailar G., Ribstein J., Mimran A. 2002. Dietary sodium and target organ damage in essential hypertension. *American Journal of Hypertension*, 15, 222-229.

FSAI. 2005. Salt and health: review of the scientific evidence and recommendations for public policy in Ireland. Food Safety Authority of Ireland, Dublin.

Gerić T., Lilić S., Babić Milijašević J., Vranić D., Jovanović J., Baltic T., Borović B. 2021. Dietary salt/sodium intake through consumption of animal origin foodstuffs available on the Serbian market. *Meat Technology*, 62(1), 33–40.

Gimeno O., Astiasarán I., Bello J. 1998. A mixture of potassium, magnesium and calcium chlorides as a partial replacement of sodium chloride in dry fermented sausages. *Journal of Agricultural and Food Chemistry*, 46, 4372–4375.

Gimeno O., Astiasarán I., Bello J. 2001. Influence of partial replacement of NaCl with KCl and $CaCl_2$ on microbiological evolution of dry fermented sausages. *Food Microbiology*, 18, 329–334.
Haddy F.J. 2006. Role of dietary salt in hypertension. *Life Sciences*, 79, 1585-1592.
He F.J., MacGregor G.A. 2009. A comprehensive review on salt and health and current experience of worldwide salt reduction programmes. *Journal of Human Hypertension*, 23(6), 363-384.
Hernández Correas N., Abellán A., Cayuela J. M., Bande-De León C., Tejada L. 2024. Effect of overripening on the physico-chemical and sensory characteristics of boneless, salt-reduced Iberian dry-cured ham. *Foods*, 13*(*10), 1588.
Hunter W.R., Dhaun N, Bailey A.M. 2022. The impact of excessive salt intake on human health. Nature Reviews Nephrology, 18, 321-335.
Joffres M., Campbell N., Manns B., Tu K. 2007. Estimate of the benefits of a population-based reduction in dietary sodium additives on hypertension and its related health care costs in Canada. *Canadian Journal of Cardiology*, 23, 437–443.
León-Crespo F., Galán-Soldevilla H., Peralta-Fernández A., Ciudad-González N., Balderas-Zubeldia B., Céspedes-Sánchez F., Martín-Serrano A., Torres-Muńoz M.C. 1997. La salazón del jamón: bases para una tecnología racional. *Cárnica, 2000*, July, 33–51.
Lilić S., Borović B., Vranić D. 2014. Mogućnosti supstitucije natrijum-hlorida nekim hloridnim solima u procesu proizvodnje suvog svinjskog mesa. *Tehnologija mesa*, 55, 2, 176-183.
Lilić S., Vranić D. 2015. Promene aktivnosti vode i sadržaja natrijuma, kalijuma, magnezijuma i kalcijuma u svinjskom mesu salamurenom različitim hloridnim solima. *Tehnologija mesa*, 56(2), 137-143.
Lilić S., Babić J., Borović B., Spalević Lj., Maslić-Strižak D., Pavlović M., Milijašević M. 2016. Effect of sodium chloride reduction in dry pork on sensory quality parameters and instrumentally measured colour. *Biotechnology in Animal Husbandry*, 32(4), 383-391.
Lorenzo J.M., Bermúdez R., Domínguez R., Guiotto A., Franco D., Purriños L. 2015. Physicochemical and microbial changes during the manufacturing process of dry cured lacon salted with potassium, calcium and magnesium chloride as sa partial replacement for sodium chloride. *Food Control*, 50, 763–769.
MacGregor G.A., De Wardener H.E. 1997. Idiophatic edema. In: Schrier R. W., Gottschalk C.W., eds. *Diseases of the Kidney*. Little Brown and Company, Boston.
Mickleborough T.D., Lindley M.R., Ray S. 2005. Dietary salt, airway inflammation and diffusion capacity in exercise-induced asthma. *Medicine and Science in Sports and Exercise*, 37(6), 904-914.

Muñoz-Rosique B., Hernández-Correas N., Abellán A., Bueno E., Gómez R., Tejada L. 2023. Influence of pig genetic line and salt reduction on peptide production and bioactivity of dry-cured hams. *Foods*, 12(5), 1022.

Murray C.J.L., Lauer J.A., Hutubessy R.C.W., Niessen L., Tomijima N., Rodgers A., Lawes C.M.M., Evans D.B. 2003. Effectiveness and costs of interventions to lower systolic blood pressure and cholesterol. *The Lancet*, 361, 717–725.

Perry I.J., Beevers D.G. 1992. Salt intake and stroke: a possible direct effect. *Journal of Human Hypertension*, 6, 23-25.

Prändl O. 1988. Verarbeitung des Fleisches, Grundlagen der Haltbarmachung, Fleisch: Technologie und Hygiene der Gewinnung und Verarbeitung, Stuttgart: Ulmer, 234–372.

Purriños L., Franco D., Carballo J., Lorenzo J. M. 2012. Influence of the salting time on volatile compounds during the manufacture of dry-cured pork shoulder "lacón". *Meat Science*, 92, 627-634.

Purriños L. 2015. Physicochemical and microbial changes during the manufacturing process of dry-cured lacón salted with potassium, calcium and magnesium chloride as a partial replacement for sodium chloride. *Food Control*, 50, 763-769.

Reducing Salt Intake: What's Happening Around the World. 2024. Available at: https://khni.kerry.com/articles/nutrition-regulations-policies/reducing-salt-intake-whats-happening-around-the-world/. Accessed 2 April 2026.

Rico E., Toldrá F., Flores J. 1991. Effect of dry-cured process parameters on pork muscle cathepsin B, H, and L activities. Zeitschrift fur Lebensmittel-Untersuchung und-Forschung, 193, 541–544.

Roberts W.C., Roberts W.C. 2001. Facts and ideas from anywhere. *Proceedings Baylor University Medical Center*, 14(3), 314-322.

Schmieder R.E., Messerli F.H. 2000. Hypertension and the heart. *Journal of Human Hypertension*, 14, 597–604.

Selmer R.M., Kristiansen I.S., Haglerød A., Graff-Iversen S., Larsen H.K., Meyer H.E., Bonaa K.H., Thelle D.S. 2000. Cost and health consequences of reducing the population intake of salt. *Journal of Epidemiology & Community Health*, 54, 697–702.

Toldrá F., Rico E., Flores J. 1993. Cathepsin B, D, H and L activities in the processing of dry-cured ham. *Journal of the Science of Food and Agriculture*, 62, 157–161.

Tsugane S., Sasazuki S., Kobayashi M., Sasaki S. 2004. Salt and salted food intake and subsequent risk of gastric cancer among middle-aged Japanese men and women. *British Journal of Cancer*, 90, 128–134.

Ventanas J., Cava R. 2001. Dinámica y control del proceso de secado del jamón ibérico en secaderos y bodegas naturales y en cámaras climatizadas. In: *Tecnología del jamón ibérico. De los sistemas tradicionales a la explotación racional del sabor y el aroma* (255-293). Mundi-Prensa, Madrid.

WHO. 2014. Fact sheed N°393, Salt reduction. Available at: http://www.who.int/mediacentre/factsheets/fs393/en/. Accessed 2 April 2026.

Wu H., Zhang Y., Long M., Tang J., Yu X., Wang J. et al. 2014. Proteolysis and sensory properties of dry-cured bacon as affected by the partial substitution of sodium chloride with potassium chloride. *Meat Science*, 96, 1325-1331.

Xiang Y., Lin X., Zheng M., Deng K., Miao S., Zheng B., Zhang L. 2025. Salt reduction in processed meat products: Integrated strategies and mechanisms to maintain saltiness perception. *Trends in Food Science & Technology*, 166, 105420.

Xiong Y.L., Brekke C.J. 1991. Gelation properties of chicken myofibrils treated with calcium and magneisum chlorides. *Journal of Muscle Foods*, 21-36.

Yamanaka H., Akimoto M., Sameshima T., Arihara K., Itoh M. 2005. Effects of bacterial strains on the development of the ripening flavor of cured pork loins. *Animal Science Journal*, 76, 499-506.

Received 6 April 2026; accepted for publication 18 May 2026

Biotechnology in Animal Husbandry 42(1), 47-59, 2026
Publisher: Institute for Animal Husbandry, Belgrade-Zemun
ISSN 1450-9156
UDC 575.113'636.588
https://doi.org/10.2298/BAH2601047O

GENETIC DIVERSITY OF PROLACTIN GENE IN NIGERIAN FULANI AND YORUBA CHICKEN ECOTYPES

Osamede Henry Osaiyuwu[1], Olubunmi Elizabeth Malomo[1], Ismaila Muritala[2]

[1] University of Ibadan, Department of Animal Science, Ibadan, Oyo State, Nigeria.
[2] University of Zululand, Department of Agriculture, KwaDlangezwa, KwaZuluNatal, South Africa
Corresponding author: Ismaila Muritala, muritalaI@unizulu.ac.za
Original scientific paper

Abstract: Understanding genetic diversity of functional genes is a prerequisite to improving selection and preserve the indigenous animal breeds, known for adaptive traits and ability to utilize low quality feed. This study evaluated variation in the prolactin (PRL) gene, an essential regulator of reproductive characteristics, in two prominent Nigerian indigenous chicken ecotypes: Fulani and Yoruba. Blood samples were collected from 100 local chicken across six geographical locations in Oyo State, Nigeria. Genomic DNA was isolated for PCR amplification of a 24-bp insertion/deletion in the PRL gene. Allele and genotype frequencies, heterozygosity, fixation indices, and Hardy-Weinberg equilibrium (HWE) were assessed using conventional population genetics methodologies. Two alleles, insertion and deletion (A and B, respectively) and three genotypes (AA, AB, BB) were characterized using bands size on the gel electrophoresis. Although allele frequencies were comparable in both ecotypes, Fulani hens demonstrated more heterozygosity and agreed with Hardy-Weinberg equilibrium, indicating genetic stability. Yoruba hens exhibited higher inbreeding and divergence from HWE ($p<0.05$). Genetic distance and differentiation values demonstrated significant similarity between the ecotypes, which is likely resulting from unrestrained mating. These findings show the need for organized breeding programs to preserve genetic integrity and utilize indigenous genetic resources.

Key words: mating, gene, electrophoresis, genetic diversity

Introduction

The sustainability and effective management of indigenous animal as a genetic resource depend on a clear understanding of genetic variability among the locally adapted livestock breeds (Vanvanhossou et al., 2021). In many developing countries, Nigeria included, indiscriminate mating and inadequate genetic management have led to an increase inbreeding rate and the erosion of essential genetic resources (Martyniuk, 2021). Endris et al. (2023) showed that the loss of local breeds may occur through breed replacement or dilution through crossbreeding. These concerns are very important in poultry farming, where local chicken plays a significant role in rural food security, economic growth, and cultural legacy. Indigenous chickens are valued for their adaptability, disease resistance, low management needs, and socio-economic significance (Singh et al., 2023).

Crossbreeding indigenous hens with an exotic rooster weakens the thermotolerance and disease resistance in the resulting offspring (Kpomasse et al., 2023). The Fulani and Yoruba ecotypes in Nigeria are among the most prevalent and economically important local chicken populations (Sanusi and Oseni, 2020). Typically reared in vast scavenging systems by smallholder farmers, particularly women, these ecotypes are essential for supporting rural livelihoods. Notwithstanding their potential, these indigenous avian species are inadequately used in formal breeding initiatives, and their genetic diversity remains incompletely delineated. Molecular markers serve as efficient instruments for assessing genetic diversity and identifying potential genes associated with production and reproductive characteristics (Kumar et al., 2024). One of such genes is prolactin (PRL), which encodes a hormone integral to essential reproductive functions, including broodiness, oviposition, follicular maturation, and parental care (Hu et al., 2017; Mo et al., 2022).

Polymorphisms in the PRL gene, especially within regulatory and intronic regions, have been linked to age at first lay, clutch size, and incubation behaviour in chicken breeds (Chakraborty and Saha, 2021). Whereas the influence of PRL polymorphisms on trait expression is extensively documented in selectively bred populations (El-Magd et al., 2021), there is a significant paucity of data for PRL gene diversity in African indigenous chickens. Nigerian Fulani and Yoruba chickens, due to their unique evolutionary history influenced by natural selection and conventional breeding methods (Rachman et al., 2024), may possess discrete genetic variants of the PRL gene pertinent to productivity and adaptability. However, given the absence of fundamental genetic data, attempts to formulate focused breeding techniques remain speculative. This study examines the genetic diversity of the PRL gene in Nigerian Fulani and Yoruba chicken ecotypes at a 24-base pair insertion/deletion (Indel) marker to resolve this gap. The study seeks to provide useful data for conservation planning and the formulation of marker-assisted selection strategies specific to indigenous chicken development.

Materials and Methods

Ethical approval was obtained from the animal research ethics committee of the University of Ibadan, Nigeria and the experiment was conducted according to the approved protocol and standard set by the committee.

Sample collection

Blood samples were randomly collected via jugular venepuncture from 100 birds, equally sourced from each ecotype across six distinct locations in Oyo State, Nigeria (Figure 1). The blood samples were stained on FTA cards and maintained at room temperature until further analysis (Murital et al., 2015).

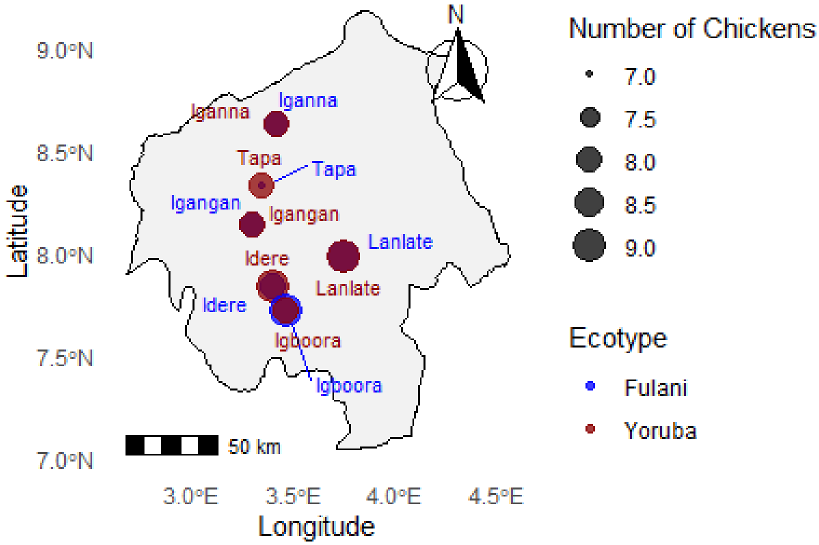

Figure 1. Spatial distribution of the sampled animals in Oyo State, Nigeria

DNA Isolation and quantification

Five pouches from the blood-stained FTA card were taken and transferred into a 200 µl PCR tube. One hundred and fifty microliters of Tris 0.1% SDS buffer was introduced to the samples in the PCR tube and agitated for thirty minutes to disrupt the cell membrane. The supernatant was thereafter discarded with minimum disturbance to the residue at the base of the PCR tube. Distilled water (150µl) was used to rinse the residue through agitation. The supernatant was subsequently extracted with a micropipette. Subsequently, an additional 150µl of distilled water was introduced to the residue and incubated for 10 minutes, after which the supernatant was discarded. A volume of 50µl of distilled water was added to the residual and incubated at 90ºC in a thermal-cycler (Bio-system veritiTM Gene Amp (R)) for an additional 15 minutes. The extracted DNA was evaluated for quality and quantity using 1% agarose gel electrophoresis and a spectrophotometer. The DNA was preserved at -20ºC for subsequent examination. This was later amplified and genotyped using insertion and deletion alleles— allele A is with the insertion and allele B with the deletion.

Polymerase chain reaction procedure

The amplification of PRL-24-bp Indel (np 358) region using primers (Forward PRL primer -5′-TTT AAT ATT GGT GGG TGA AGA GAC A-3′ and Reverse PRL primer-5′ -ATG CCA CTG ATC CTC GAA AAC TC-3′) described by Cui et al. (2006), in a polymerase chain reaction (PCR) using a thermal-cycler (ABI9700). The PCR master mix (Thermo-scientific) contained 6.25 U of FastStart™ Taq DNA Polymerase (5 U/µl; Roche Diagnostics), about 10-20 ng of DNA template (0.5µl), 5 pmol of each of the forward and reverse primers (0.25µl) and 4µl of Nuclease free water to form 6.25µl solution in each PCR tubes and positioned in the interchangeable blocks of 16 wells of 0.2 ml. with an initial incubation and enzyme activation condition at 94°C for 5 min; followed by 35 cycles of 30 sec at 94°C, 30 sec at 54°C, and 30 sec at 72°C and a final extension at 72°C for 5 min.

Allele determination

For the characterization of the region of interest, the PCR product was mixed with 2 µl of SYBR Green dye and then transferred directly onto a 3% agarose gel for electrophoresis, alongside a standard DNA ladder (100 bp). A UV transilluminator was used to visualize the bands associated with PCR products. The dimensions of the PCR products were assessed based on band migration relative to the molecular weight of the ladder, as described by Kumar et al. (2021), and were used for the characterization of the alleles at the PRL-24-bp Indel (np 358) region.

Statistical analysis

Frequencies of alleles and genotypes, heterozygosity, genetic distance, inbreeding coefficient within subpopulation (F_{IS}), inbreeding coefficient within the entire population (F_{IT}), fixation index (F_{ST}), gene flow and their fit to Hardy-Weinberg equilibrium were estimated using POPGENE (Version. 1.31) software (Yeh et al., 1997). Genetic distance and identity were enumerated according to Nei (1978). Level of significance ($p<0.05$) was evaluated using the 5,000 permutations of the studied population (Che et al., 2014). Distance-based clustering (average-linkage) was conducted on a normalized genotype-distance matrix to show sample linkages (Borgsmüller et al., 2024). Analysis of molecular variance (AMOVA) was computed to measure the variance components between subpopulations and within subpopulations (Excoffier et al., 1992).

Results and Discussion

PRL gene polymorphism and genotype frequencies

This study provides one of the first comparisons of PRL gene polymorphism in Nigerian indigenous chicken ecotypes. Genotyping of the 24-bp insertion/deletion (Indel) in a locus on the PRL gene showed the presence of two alleles: insertion (A) and deletion (B), resulting in three genotypes: AA (insertion/insertion), AB (insertion/deletion), and BB (deletion/deletion) (Figure 2). A similar report was made by Eichie et al. (2016) where 2 alleles and 3 genotypes were found in Nigerian quail strains. Previously, 2 alleles and 3 genotypes were reported in Nigerian quail strains (Eichie et al., 2016). Also, insertion/deletion polymorphisms were recorded in indigenous and commercial chickens by Cui et al. (2006). Meanwhile Allele frequencies in entire population were similar for the two recorded alleles (A = 0.50 and B = 0.50; Table 1). However, stratification of the allele frequency by ecotype showed that the Fulani chickens had 0.51 and 0.49 for alleles A and B, respectively while Yoruba chickens had frequencies of 0.49 of 0.51 of A and B, respectively. While the overall genotype frequencies were 0.32; 0.36 and 0.32 for genotypes AA, AB and BB, respectively. The ecotype-specific analysis showed that Fulani chickens had AA = 0.31, AB = 0.40, BB = 0.29 and Yoruba chickens had AA = 0.33, AB = 0.33, BB = 0.35. The allelic frequencies observed in the Fulani and Yoruba chickens show a balanced genetic makeup, which reflects a conserved polymorphic structure of the PRL gene in Nigerian ecotypes. The moderate genotype diversity, especially the relatively high observed heterozygote in Fulani chickens, supports the existence of functional genetic variation, which is important for adaptive traits such as reproduction and survival.

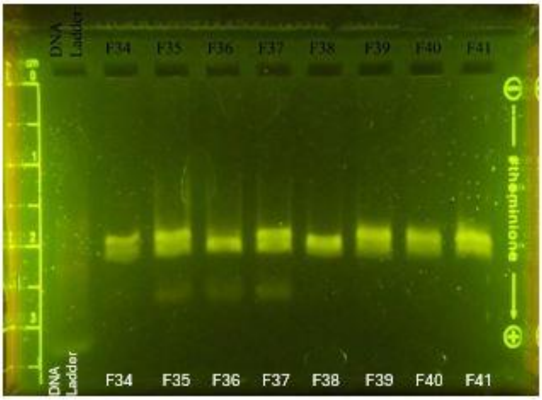

Figure 2. Gel electrophoresis showing the PRL fragment bands (alleles) from Fulani and Yoruba chicken ecotypes

Table 1. Allele and genotypic frequencies of PRL gene in Fulani and Yoruba chicken ecotypes

Breed	Allele Frequency		Genotype Frequency		
	A	B	AA	AB	BB
Fulani	0.51	0.49	0.31	0.40	0.29
Yoruba	0.49	0.51	0.33	0.33	0.35
Overall	0.50	0.50	0.32	0.37	0.32

A-insertion allele; B-deletion allele; AA-insertion-insertion; AB-insertion-deletion; BB-deletion-deletion.

Hardy-Weinberg equilibrium and heterozygosity

The Fulani ecotype exhibited higher observed heterozygosity (H_o) and conformed to Hardy-Weinberg equilibrium (HWE), indicating a stable genetic structure (Table 2). In contrast, the Yoruba ecotype showed a lower H_o and a deviation from HWE, indicating a heterozygote deficit. This was corroborated by higher F_{IS} estimated for the Yoruba chickens (Table 3), suggesting potential inbreeding or non-random mating patterns in that ecotype. The higher heterozygosity and compliance with Hardy-Weinberg equilibrium in Fulani chickens may be attributed to random mating patterns and reduced artificial selection pressure, thereby maintaining a genetically stable population (Luo et al., 2025; Calò et al., 2021). On the other hand, the deficit of heterozygotes and deviation from HWE in the Yoruba chickens suggests the influence of inbreeding or localized non-random mating. This aligns with reports by Davila et al. (2009), who observed reduced heterozygosity in Spanish hens due to selective breeding practices focused on morphological traits. Higher heterozygosity observed in the Fulani ecotype, together with its conformity to HWE, may also be attributed to the traditional nomadic production system under which these chickens are managed. Under such free-range, village-based systems, uncontrolled mating and frequent inter-flock interactions enhance gene flow and promote random mating, thereby maintaining genetic diversity. This is particularly relevant for the prolactin gene, a key regulator of broodiness and reproductive behaviour in chickens (Jiang et al., 2025). However, selective breeding of highly valued chickens by Yoruba ecotype farmers imposes selection pressure that may explain the observed deviation from Hardy–Weinberg equilibrium.

Table 2. Heterozygosity and Chi-square (X^2) for Hardy-Weinberg Equilibrium (HWE) tests at PRL gene in Fulani and Yoruba chicken ecotypes

Breed	Ne	*Ho*	*He*	F_{IS}	X^2	P value	HWE
Fulani	2.00	0.40	0.51	0.21	2.29	0.13	
Yoruba	2.00	0.33	0.51	0.35	5.58	0.02	*
Overall	2.00	0.37	0.51	0.28	7.15	0.01	*

Table 3. Summary of genetic distance, genetic identity, fixation indices (F_{IT}, F_{IS} and F_{ST}) and gene (Nm) flow for overall populations

Locus	Genetic distance	Genetic diversity	F_{IS}	F_{IT}	F_{ST}	NM*
PRL	0.0010	0.9990	0.28	0.28	0.0005	499.75

***NM = gene flow estimates from $F_{ST} = 0.25\ (1- F_{ST})\ /\ F_{ST}$

Phylogenetic analysis using the neighbour-joining method (Figure 3) confirmed the considerable genetic similarity between the two ecotypes. The clustering pattern demonstrated the lack of divergence at the PRL gene region considered in this study between the Fulani and Yoruba chicken ecotypes. The branch lengths correspond to genetic distance, and clustering did not reveal clear ecotype separation (Figure 3). This corresponds with low estimated Gst (Figure 4), and a permutation test (5,000 replicates) confirmed that the observed value was not statistically significant (($p>0.05$); Figure 4). The result from analysis of variance (AMOVA), shows that most variation (~98-99%) was within populations, while only a small proportion (~1-2%) was attributable to differences between Fulani and Yoruba chickens (Table 4). The phylogenetic clustering further illustrates the genetic similarity between individuals from the two ecotypes, reinforcing the notion of limited divergence at the PRL locus. This may reflect a recent common ancestry or ongoing genetic exchange, which maintains allelic continuity across ecotypes. Such a pattern suggests that PRL gene variation is preserved across Nigeria's indigenous chickens, a valuable insight for marker-assisted selection and breeding strategies aimed at improving productivity and stress adaptability. The findings indicate moderate PRL gene diversity within each ecotype; however minimal genetic divergence exists between them. This shows the need for the establishment of localized and ecotype-specific breeding programs that can promote preserving essential adaptive features. The PRL gene offers information on genetic diversity associated with reproduction, however it constitutes merely a little portion of the genome. For better understanding of the genetic composition and adaptive feature of indigenous Nigerian chickens, further research should employ high-throughput genotyping methods (e.g., SNP arrays, Genome-Wide-Association Study). These should be integrated with phenotypic performance data to uncover functional markers linked to productivity, disease resistance/resilience, and environmental adaptability.

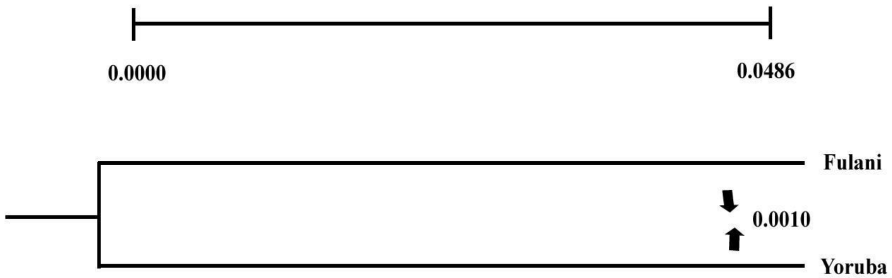

Figure 3. Phylogenetic relationship between Fulani and Yoruba chicken ecotypes based on genotype distances.

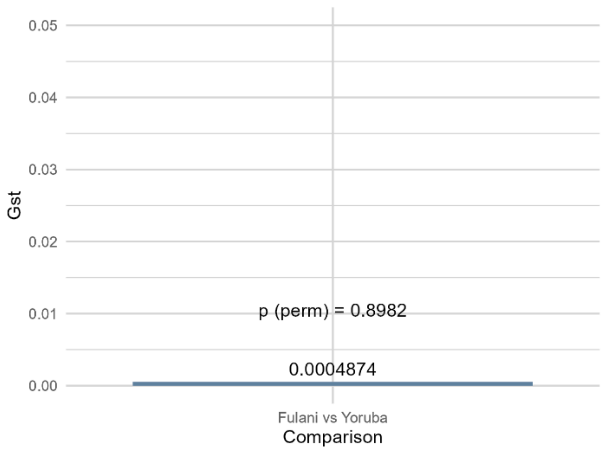

Figure 4. Genetic differentiation index (Gst) between populations

Table 4. Variance partitioning from analysis of molecular variance (AMOVA)

Source of Variation	Df	Sum of Squares (SS)	Variance Components (σ²)	Percentage Variation (%)
Among populations	1	~0.02	~0.0005	~1–2%
Within populations	89	~44.00	~0.49	~98–99%
Total	90	~44.02	~0.4905	100%

Conclusion

Genetic diversity at PRL-24-bp Indel (np 358) region of the prolactin (PRL) gene in the Fulani and Yoruba indigenous chicken ecotypes of Nigeria were analysed. The characterization of two alleles (A and B) and three genotypes (AA, AB, and BB), along with approximately equal allele and genotype frequencies, signifies modest genetic variation in both ecotypes. The Fulani ecotype showed higher heterozygosity and in agreement with Hardy-Weinberg equilibrium (HWE), indicating a more genetically stable population, whereas the Yoruba ecotype showed higher inbreeding level and deviations from HWE. However, the genetic differentiation between the two ecotypes was negligible. As shown by low F_{ST} and genetic distance metrics. The findings highlight the importance of developing ecotype-specific breeding strategies to promote genetic diversity, thereby providing a valuable genetic resource for resilience against disease outbreaks, climate change, and feed shortages in the future.

Genetska raznolikost gena za prolaktin kod nigerijskih ekotipova pilića Fulani i Joruba

Osamede Henri Osaijuvu, Olubunmi Elizabeth Malomo, Ismaila Muritala

Rezime

Razumevanje genetske raznolikosti funkcionalnih gena je preduslov za poboljšanje selekcije i očuvanje autohtonih rasa životinja, poznatih po adaptivnim osobinama i sposobnosti korišćenja hrane niskog kvaliteta. Ova studija je procenila varijacije u genu za prolaktin (PRL), esencijalnom regulatoru reproduktivnih karakteristika, kod dva istaknuta nigerijska autohtona ekotipa pilića: Fulani i Joruba. Uzorci krvi su prikupljeni od 100 lokalnih pilića na šest geografskih lokacija u državi Ojo, Nigerija. Genomska DNK je izolovana za PCR amplifikaciju insercije/delecije od 24 bp u PRL genu. Frekvencije alela i genotipa, heterozigotnost, indeksi fiksacije i

Hardi-Vajnbergova ravnoteža (HWE) procenjeni su korišćenjem konvencionalnih metodologija populacione genetike. Dva alela, insercija i delecija (A i B, respektivno) i tri genotipa (AA, AB, BB) okarakterisani su korišćenjem veličine fragmenata na gel elektroforezi. Iako su frekvencije alela bile uporedive u oba ekotipa, kokoške rase Fulani pokazale su veću heterozigotnost i bile su u Hardi-Vajnbergovoj ravnoteži, što ukazuje na genetsku stabilnost. Kokoške rase Joruba pokazale su veći stepen inbridinga i divergencije od HWE ($p<0{,}05$). Genetska udaljenost i vrednosti diferencijacije pokazale su značajnu sličnost između ekotipova, što je verovatno rezultat neograničenog parenja. Ovi nalazi ukazuju na potrebu za organizovanim programima uzgoja kako bi se očuvao genetski integritet i iskoristili autohtoni genetski resursi.

Ključne reči: parenje, gen, elektroforeza, genetska raznolikost

Author Contributions: O.H.O. conceived and designed the analysis; E.M.O. collected the data; I.M. contributed data or analysis tools; E.M.O. performed the analysis; E.M.O. and I.M. wrote the paper.

Acknowledgments and funding

The authors acknowledge Department of Animal Science, University of Ibadan, Oyo State, Nigeria for providing the farm and laboratory facilities for this research. This research was funded by parents of the postgraduate student.

Conflicts of interest

The authors declare no conflict of interest.

Statement of the AI tool use

During the preparation of this paper, the authors used ChatGPT to edit the English grammar and spelling. After using this tool, the authors reviewed and edited the content as necessary and take full responsibility for the content of the published article.

References

Bataillon T., Gauthier P., Villesen P., Santoni S., Thompson J. D., Ehlers B.K. 2022. From genotype to phenotype: Genetic redundancy and the maintenance of an adaptive polymorphism in the context of high gene flow. *Evolution Letters*, 6(2), 189-202. https://doi.org/10.1002/evl3.277

Borgsmüller N., Kuipers J., Gawron J., Roncador M., Pohly M., Acar E., Do T.H.L., Reisenauer S., Feldkamp M.J., Beisel C., Zenz T., Moor A., Beerenwinkel N. 2024. DemoTape: Computational demultiplexing of targeted single-cell sequencing data. bioRxiv 2024.12.06.627152. https://doi.org/10.1101/2024.12.06.627152

Calò C.M., Onali F., Robledo R., Flore L., Massidda M., Francalacci P. 2021. Worldwide variation of the COL14A1 gene is shaped by genetic drift rather than selective pressure. *Molecular genetics & genomic medicine*, 9(4), e1629. https://doi.org/10.1002/mgg3.1629

Chakraborty A., Saha I. 2021. Regulatory effects of prolactin on breeding and migratory behaviours in birds. *South Asian Journal of Experimental Biology*, 11(3), 337-344. https://doi.org/10.38150/sajeb.11(3).p337-344

Che R., Jack J.R., Motsinger-Reif A.A., Brown C.C. 2014. An adaptive permutation approach for genome-wide association study: evaluation and recommendations for use. *BioData Mining*, 7(1), 9. https://doi.org/10.1186/1756-0381-7-9

Cui J.X., Du H.L., Liang Y., Deng X.M., Li N., Zhang,X.Q. 2006. Association of polymorphisms in the promoter region of chicken prolactin with egg production. *Poultry Science*, 85:26-31. https://doi.org/10.1093/ps/85.1.26

Dávila S.G., Gil M.G., Resino-Talaván P., Campo J.L. 2009. Evaluation of diversity between different Spanish chicken breeds, a tester line and a White Leghorn population based on microsatellite markers. *Poultry Science*, 88: 2518-2525. https://doi.org/10.3382/ps.2009-00347

Eichie F.O., Salaoko A.E., Aggrey S.E. 2016. Genetic Diversity of Prolactin Gene in two strains of Japanese quail (Coturnix Coturnix Japonica) in Nigeria. *International Journal of Poultry Science*, 15: 349-357. https://doi.org/10.3923/ijps.2016.349.357

El-Magd M.A., Fathy A., Kahilo K.A., Saleh A.A., El Sheikh A.I., Al-Shami S., El-Komy S.M. 2021. Polymorphisms of the PRLR gene and their association with milk production traits in Egyptian Buffaloes. *Animals*, 11(5), 1237. https://doi.org/10.3390/ani11051237

Endris M., Hosseini S.M., Tadesse Y., Abate M. 2023. Genetic Management in Conservation Programs: A Review. *Global Journal of Animal Scientific Research*, 11(3), 67-88.

Excoffier L., Smouse P.E., Quattro J.M. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, 131(2), 479-491. https://doi.org/10.1093/genetics/131.2.479

He Z.Z., Stotz G.C., Liu X., Liu J.J., Wang Y.G., Yang J., Li L.F., Zhang W.J., Nan P., Song Z.P. 2024. A global synthesis of the patterns of genetic diversity in endangered and invasive plants. *Biological Conservation*, 291:110473. https://doi.org/10.1016/j.biocon.2024.110473

Hu S., Duggavathi R., Zadworny D. 2017. Regulatory Mechanisms Underlying the Expression of Prolactin Receptor in Chicken Granulosa Cells. *PLoS ONE*, 12(1):e0170409. https://doi.org/10.1371/journal.pone.0170409

Jiang R.S., Xu G.Y., Zhang X.Q., Yang N. 2005. Association of polymorphisms for prolactin and prolactin receptor genes with broody traits in chickens. *Poultry Science*, 84(6), 839–845. https://doi.org/10.1093/ps/84.6.839

Johnson J.A., Athrey G., Anderson C.M., Bell D.A., Dixon A., Kumazawa Y. et al. 2023. Whole genome survey reveals extensive variation in genetic diversity and inbreeding levels among peregrine falcon subspecies. *Ecology and Evolution*, 13(7), e10347. https://doi.org/10.1002/ece3.10347

Kpomasse C.C., Kouame Y.A.E., N'nanle O., Houndonougbo F.M., Tona K., Oke O.E. 2023. The productivity and resilience of the indigenous chickens in the tropical environments: improvement and future perspectives. *Journal of Applied Animal Research*, 51(1), 456-469. https://doi.org/10.1080/09712119.2023.2228374

Kumar R., Das S.P., Choudhury B.U., Kumar A., Prakash N.R., Verma R. et al. 2024. Advances in genomic tools for plant breeding: harnessing DNA molecular markers, genomic selection, and genome editing. *Biological Research*, 57(1), 80. https://doi.org/10.1186/s40659-024-00562-6

Kumar S.J., Susmita C., Agarwal D.K., Pal G., Rai A.K., Simal-Gandara J. 2021. Assessment of genetic purity in rice using polymorphic SSR markers and its economic analysis with grow-out-test. *Food Analytical Methods*, 14, 856-864. https://doi.org/10.1007/s12161-020-01927-9

Luo J., Dai X., Chen J., Shuang H., Yuan C., Luo D. 2025. Study on the characteristics of genetic diversity and population structure of a rare and endangered species of Rhododendron nymphaeoides (*Ericaceae*) based on microsatellite markers. *BMC Plant Biology*, 25(1), 310. https://doi.org/10.1186/s12870-025-06362-8

Martyniuk E. 2021. Policy effects on the sustainability of animal breeding. *Sustainability*, 13(14), 7787. https://doi.org/10.3390/su13147787

Mo G., Hu B., Wei P., Luo Q., Zhang X. 2022. The Role of Chicken Prolactin, Growth Hormone and Their Receptors in the Immune System. *Frontiers in Microbiology*,13:900041. https://doi.org/10.3389/fmicb.2022.900041

Murital I., Afolayan O., Bemji M.N., Dadi O., Landi V., Martínez A., Delgado J.V., Adebambo O.A., Aina A.B.J., Adebambo A.O. 2015. Genetic diversity and population structure of Nigerian indigenous goat using DNA microsatellite markers. *Archivos de zootecnia*, 64(246), 93-98. https://doi.org/10.21071/az.v64i246.382

Nei M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics,* 89: 583-590. https://doi.org/10.1093/genetics/89.3.583

Petit R.J., Excoffier L. 2009. Gene flow and species delimitation. *Trends in Ecology & Evolution*, 24(7), 386-393. https://doi.org/10.1016/j.tree.2009.02.011

Rachman M.P., Bamidele O., Dessie T., Smith J., Hanotte O., Gheyas A.A. 2024. Genomic analysis of Nigerian indigenous chickens reveals their genetic diversity and adaptation to heat-stress. *Scientific Reports*, 14(1), 2209. https://doi.org/10.1038/s41598-024-52569-4

Rashidi H., Rahimi-Mianji G., Farhadi A., Gholizadeh M. 2012. Association of prolactin and prolactin receptor gene polymorphisms with economic traits in breeder hens of indigenous chickens of Mazandaran province. *Iran Journal of Biotechnology*, 10: 129-135.

Sanusi A., Oseni S. 2020. Nigerian Fulani ecotype chickens: Growth performance under two production systems. *Genetics & Biodiversity Journal*, 4(1), 14-21. https://doi.org/10.46325/gabj.v4i1.71

Singh M., Patton R.N., Mollier R.T., Pongener N., Yadav R., Singh V. et al. 2023. Indigenous chicken production system in different agro-ecology of Indian Himalayan Region: implication on food and economic security. *Frontiers in Nutrition*, 10:1244413. https://doi.org/10.3389/fnut.2023.1244413

Vanvanhossou S.F.U., Dossa L.H., König S. 2021. Sustainable management of animal genetic resources to improve low-input livestock production: insights into local Beninese cattle populations. *Sustainability*, 13(17), 9874. https://doi.org/10.3390/su13179874

Yeh F.C., Yang R.C., Boyle T.B., Ye Z.H., Mao J.X. 1997. PopGene, the user-friendly shareware for population genetic analysis. Molecular biology and biotechnology centre, University of Alberta, Canada, 10, 295-301.

Received 10 March 2026; accepted for publication 26 May 2026

Biotechnology in Animal Husbandry 42(1), 61-75, 2026
Publisher: Institute for Animal Husbandry, Belgrade-Zemun

ISSN 1450-9156
UDC 637.07'637.433
https://doi.org/10.2298/BAH2601061P

THE IMPACT OF STORAGE TEMPERATURE AND DURATION ON THE MICROBIOLOGICAL SAFETY, QUALITY, AND SHELF-LIFE OF BANAT NAKED NECK HEN EGGS BEFORE EXPIRATION

Maja Petričević[1], Maša Radojičić[1], Veselin Petričević[1], Tamara Stamenić[1], Tanja Keškić[1], Slobodan Dolašević[1], Zlata Kralik[2]

[1] Institute for Animal Husbandry, Autoput Beograd-Zagreb 16, 11080 Belgrade, Serbia
[2] Faculty of Agrobiotechnical Sciences Osijek, Josip Juraj Strossmayer University of Osijek, V. Preloga 1, 31000 Osijek, Croatia
Corresponding author: Maja Petričević, majanovakovic@live.com
Original scientific paper

Abstract: Temperature is a major determinant of egg quality loss, but data for Banat Naked Neck hens are limited. This study evaluated 240 freshly laid eggs stored for 28 days at room temperature (RT, 20–22°C) or under refrigeration (RF, 4–6°C). Egg mass, shell deformation and breaking strength, shell weight and thickness, albumen height, Haugh units (HU), yolk colour, and albumen and yolk pH were measured on days 1, 7, 14, 21, and 28; preliminary screening for determine the total bacterial count, *Salmonella* spp. and *Listeria monocytogenes* was also performed. The egg mass remained stable. Albumen height significantly dropped by 35.18% under RF and by 57.58% at RT when compared to the first and last day of the four-week storage period, while HU declined from 80 to 62 under RF and from 81 to 46 at RT ($p < 0.05$). Shell breaking, shell thickness and yolk colour showed no significant variation. Albumen pH increased to 9.14 under RF and 9.37 at RT ($p < 0.05$), and yolk pH increased more slowly yet followed the same temperature-dependent trend ($p < 0.05$). No target pathogens have been detected in either treatment to date. A gradual increase in total bacterial count was observed during the storage period, with a significant difference ($p<0.05$) observed on day 28. Our results show the need for the perseverance of the intact cold chain and proper storage within the recommended shelf life to preserve the optimal quality and safety of eggs.

Key words: microbiological safety, egg quality, storage temperature, sustainability, Banat Naked Neck hens

Introduction

Global egg production and consumption have been steadily increasing. According to a report by The Business Research Company (2024), the global egg market grew in 2024 compared to the previous year, and it is projected to continue growing until 2028. These data show a steady increase in egg production and consumption, suggesting that comparable trends may exist in other regions, including Serbia. According to the Statistical Office of the Republic of Serbia, the turnover of agricultural products at markets in Serbia increased by 4.5% in the first quarter of 2025 compared to the same period last year. Poultry and eggs accounted for 16.7% of the total turnover, making them one of the most significant categories alongside vegetables, milk, and fruit (https://www.stat.gov.rs)

Eggs are a valuable source of animal-derived nutrients, and with rising global demand, increased sales of this product are also expected. Their quality largely depends on the basic features—the shell, yolk, and albumen—which possess distinct nutritional and functional properties (Razi et al., 2023). Egg weight, shell strength, albumen height, yolk colour, HU, and moisture content serve as standard measures for assessing egg freshness and quality (Wengerska et al., 2023, Rho and Cho, 2024). Storage conditions greatly affect these characteristics, especially temperature and storage period. Higher temperatures and prolonged storage negatively impact quality, which is reflected in lowered albumen height and lower Haugh unit values (Kim et al., 2024). Nowaczewski et al. (2022) have reported similar results, emphasizing that extended storage leads to increased weight loss, elevated pH values of the yolk and albumen, enlargement of the air cell, and decreased albumen clarity. Obianwuna et al. (2022) also confirm these findings, highlighting that a rise in albumen pH leads to structural breakdown and reduced viscosity.

Most studies on egg quality during storage focus on eggs from hybrid laying hens, which dominate commercial production. With the development of alternative egg production systems, the question arises of how to preserve the quality of eggs from native breeds, such as the Banat Naked Neck (Škrbić et al., 2021). Previous studies have shown that this breed has potential for use in extensive and semi-extensive production systems, which can help preserve genetic diversity (Škrbić et al., 2020). For this reason, there is an increasing need to study the egg quality of such breeds in more detail, especially during storage.

While extended storage affects the physicochemical characteristics of eggs, it can also increase the risk of microbiological contamination. Keeping eggs microbiologically safe is essential for consumer health because eggs, though nutritious, become high-risk foods if they are handled or stored poorly. Harmful bacteria such as *Salmonella*, *Listeria*, and *Escherichia coli* can contaminate eggs during production, handling, transport, or storage, and thus pose a serious public-health problem (Chousalkar et al., 2021). Contaminated eggs lose quality-odor, taste, and texture changes render them unfit for consumption, and could pose particular danger (Holt et al., 2011; Hayder and Aamer, 2024).

The way eggs are handled, especially washing, further impacts microbiological safety. Although egg washing is practiced in some countries for hygienic reasons, studies have shown that it can damage the shell cuticle and thus increase the risk of contamination (Wellman-Labadie et al., 2008; Gole et al., 2014). For this reason, the European Union prohibits the washing of Class A eggs (European Commission, 2008). This regulation is based on research indicating that washing may increase spoilage and reduce microbiological stability during storage (Hutchinson et al., 2004).

Various scientific findings suggest that cleaning practices and handling methods have a significant impact on egg safety, which becomes especially important in the context of intensive global trade and increasing consumption (Zhi et al., 2016, Jones et al., 2018, Mahmoud et al., 2022). Contaminated eggs in supply chains can contribute to the spread of infections among consumers. Contamination risk varies with the product's origin, the environment in which it is produced, and how it is handled, shipped, and stored (Yamak et al., 2021). Eggs from farms, particularly in high-density and poorly ventilated environments, are at greater risk of bacterial contamination (Solis et al., 2023). Eggs stored at lower temperatures tend to have a lower contamination rate than those kept at higher temperatures (Chousalkar et al., 2021).

The performance of the safety analysis of eggs enables the detection of the critical control points in the production and distribution chain. It serves as the basis for defining measures that contribute to reducing consumer health risks (Crivei et al., 2025). Since various egg origin and their production and storage conditions can significantly affect their microbiological profile and quality, monitoring the microbiological safety of eggs from diverse sources is vital for ensuring food safety and protecting consumer health (Holt et al., 2011).

Given the limited research on preserving egg quality in autochthonous Serbian hen breeds during extended storage, this study aimed to assess how different storage conditions—simulating common household environments—affect key egg quality parameters. Similarly to other research (Gogo et al., 2021; Kim et al., 2024), selected temperatures reflect typical household refrigerator and room temperatures, which is especially relevant for consumers in moderate climates like Serbia. Particular attention was paid to microbiological safety, physicochemical properties, and indicators of freshness. These research findings were intended to contribute to a better understanding of the factors that could affect the egg quality of Banat Naked Neck hens and to advance the necessary steps that need to be taken in order to further preserve their quality over time.

Materials and Methods

The study was conducted using eggs obtained from 63-week-old Banat Naked Neck laying hens reared in a floor system. A total of 240 eggs were collected, of which 120 were allocated for quality assessment and 120 for microbiological analysis. The eggs were stored under two temperature conditions: room temperature (20–22°C) and refrigeration (4–6°C), with 60 eggs assigned to each treatment group. The refrigerated range of 4–6°C was selected to reflect typical household refrigerator temperatures. Although the Egg Quality Regulation (2019) recommends storing eggs above +5°C in commercial settings, this study focused on home storage practices, where temperatures may occasionally fall slightly below this threshold. These storage conditions were used to evaluate the impact of temperature on egg quality and microbiological safety over time. The investigation was carried out over a four-week period, with analyses performed on day 1 (fresh eggs) and at weekly intervals thereafter (days 7, 14, 21, and 28). At each time point, 12 eggs from each treatment group (i.e., 24 eggs in total per time point) were evaluated for external and internal egg quality characteristics. The same sampling schedule was used for microbiological analysis, with 12 eggs per treatment group analysed at each time point (a total of 24 eggs per time point).

Egg quality

Egg quality was evaluated immediately after collection, providing information on the initial quality of table eggs. External and internal quality traits parameters were analysed for each egg. Egg weight was determined using an electronic balance with an accuracy of ±0.02 g. Egg shell quality testing included measurements of deformation, breaking strength, weight, and thickness of the shell. Shell weight (with membranes) was determined using an electronic balance with precision ±0.02 g. Shell thickness was measured at the equator using a micrometric screw (SOMET), following membrane removal. Shell deformation was evaluated using a Marius device by applying a 500 g load to the equatorial area of the egg; results were expressed in micrometres (µm), as the mean of three measurements. Shell breaking strength was determined using a 15 kg spring at a loading speed of 70 mm/min. The obtained value was multiplied by the corresponding coefficient (k = 0.150) to express the breaking strength in kilograms. The height of the thick albumen was measured using a tripod micrometre (AMES company) at the midpoint between the yolk edge and the thick albumen, with a measurement precision of 0.1 mm. Yolk colour was assessed by visual comparison with the "Roche Yolk Colour Fan 1969", with intensity scored from 1 to 15. Egg freshness was objectively assessed by calculating HU, which combine egg weight and albumen height into a single quality index. HU were calculated employing the subsequent formula (Obianwuna et al., 2022):

$$HU = 100 \log_{10}(H - 1.7W^{0.37} + 7.6)$$

where, H represents the height of the thick albumen (in mm), and W is the egg weight (in grams).

The pH value of the albumen and yolk was measured using a pH meter (PH meter Consort C830).

Microbiological quality of eggs

Microbiological analyses were performed under aseptic conditions to minimize external contamination. For microbiological analysis, egg contents were examined. To avoid shell surface contamination, each eggshell was disinfected with 70% ethanol according to SRPS EN ISO 6887-4:2017, dried under sterile conditions, and then aseptically cracked with sterile instruments. The contents were homogenized in a 1:10 ratio with buffered peptone water prior to analysis. Total Bacterial Count (TBC) was assessed by pour plate method on Plate Count Agar and incubated at 30°C for 72 ± 3 h according to SRPS EN ISO 4833-2:2017. Upon completion of incubation, colonies were counted and expressed as CFU/mL. A representative sample was considered valid if it contained 15 to fewer than 300 colonies. The CFU/mL was calculated according to the relevant ISO standard.

Detection of *Listeria monocytogenes* was carried out according to SRPS EN ISO 11290-1:2017, which includes primary enrichment in half Fraser (LABM, England) broth (24 h at 30°C), followed by secondary enrichment in Fraser broth (24 h at 37°C), and plating onto two selective agars - ALOA (Agar Listeria according to Ottaviani and Agosti) (LABM, England) and Oxford (Biolife Italiana, Italy) agar.

To detect the presence of *Salmonella* spp., the method specified in SRPS EN ISO 6579-1:2017 was employed. Samples were first pre-enriched in non-selective medium - buffered peptone water (BPW, Biolife Italiana, Italy) for 18h at 37 °C, followed by selective enrichment in Rappaport-Vassiliadis Soya (RVS, LABM, England) broth (24 h at 41.5°C) and Müller-Kauffmann tetrathionate novobiocin (MKTTn, Biolife Italiana, Italy) broth (24 h at 37°C), and subsequently plated onto Xylose Lysine Deoxycholate (XLD, LABM, England) and Hektoen Enteric (HE, Biolife Italiana, Italy) agar at 37°C for 24 h.

Statistical analysis

The software package STATISTICA, version 12 (StatSoft Inc.), was used for statistical analysis. Two-way ANOVA analysed the results, and the significance of differences between groups was assessed at the probability level of $p<0.05$ using the Tukey post hoc test.

Results and Discussion

The external egg quality parameters, as influenced by storage temperature and duration, are summarized in Table 1. The table is structured to present results at three levels: (1) average values by temperature (RF and RT) to illustrate the main effect of storage temperature; (2) average values by storage day (1, 7, 14, etc.), regardless of temperature, to show the effect of storage duration; and (3) individual values for each treatment group and time point (e.g., RF-day 1, RT-day 1, etc.), which represent the interaction between storage temperature and duration.

The examined external quality parameters did not significantly change under the influence of temperature, storage duration, or their interaction. The egg mass did not show a significant change throughout the experiment, and a gradual decline was observed in eggs stored at both temperatures until day 28, which can be attributed to water loss through the shell pores.

Table 1. Changes in external egg quality parameters as affected by storage temperature, storage duration, and their interaction

ST (°C)	SD (day)	Egg weight (g)	Shell deformation (μm)	Breaking strength (kg)	Shell weight (g)	Shell thickness (0.01 mm)
		x̄±Sd	x̄±Sd	x̄±Sd	x̄±Sd	x̄±Sd
RF		55.70±4.68	25.80±3.44	3.85±0.41	7.18±0.65	30.50±1.50
RT		55.67±3.73	25.77±2.84	3.78±0.41	7.07±0.73	29.60±2.30
	1	56.39±3.07	24.33±2.46	3.94±0.39	7.29±0.45	30.67±1.23
	7	56.28±5.53	27.42±2.50	3.77±0.47	7.10±0.62	29.67±2.31
	14	55.77±4.99	25.00±4.49	3.90±0.38	7.11±1.13	30.00±2.70
	21	55.46±3.93	25.50±2.81	3.77±0.36	7.18±0.53	30.08±1.98
	28	54.53±3.38	26.67±2.27	3.69±0.47	6.93±0.56	29.83±1.53
RF	1	56.38±3.11	23.83±1.33	3.92±0.38	7.37±0.19	30.17±1.17
	7	56.00±7.40	27.50±2.17	3.71±0.56	7.17±0.73	29.83±1.33
	14	55.68±6.87	26.33±5.85	3.75±0.42	7.13±1.12	30.00±2.37
	21	55.63±3.06	24.67±3.27	3.92±0.44	7.32±0.56	31.67±0.52
	28	54.78±2.02	26.67±2.50	3.96±0.33	6.90±0.40	30.83±1.17
RT	1	56.40±3.32	24.83±3.31	3.96±0.43	7.22±0.64	31.17±1.17
	7	56.57±3.50	27.33±3.01	3.83±0.41	7.03±0.55	29.50±3.15
	14	55.85±2.76	23.67±2.42	4.04±0.29	7.08±1.24	30.00±3.22
	21	55.28±4.95	26.33±2.25	3.63±0.21	7.03±0.52	28.50±1.52
	28	54.27±4.58	26.67±2.25	3.42±0.44	6.97±0.73	28.83±1.17
		p value				
ST		0.984	0.967	0.474	0.563	0.071
SD		0.852	0.109	0.543	0.821	0.746
ST x SD		0.998	0.487	0.092	0.984	0.069

ST - Storage temperature; SD - Storage duration; RF - refrigeration temperature (4–6°C); RT - room temperature (20–22°C); x̄ - Average mean; Sd - Standard deviation; p>0.05 not significant

These results are consistent with the findings of Akter et al. (2014), Kumari et al. (2020), and Škrbić et al. (2021), who indicate that water evaporation increases with prolonged storage, especially at higher temperatures, leading to a reduction in egg mass.

Shell deformation changed over the storage period, but these changes seemed to leave overall egg quality unchanged. On the other hand, the shell breaking strength recorded a decrease over time, especially in eggs stored at room temperature, indicating reduced elasticity and shell strength due to moisture loss. Hagan and Eichie (2019) reported comparable results, observing that extended storage at higher temperatures weakens the shell's mechanical properties. The results of our research show that the eggshell mass did not significantly differ depending on the temperature. In contrast to our study, some authors report that storage duration at room temperature significantly affects the reduction of eggshell weight (Škrbić et al., 2021). Kanasuah et al. (2025) suggest that storage at room temperature did not significantly affect the external qualities of eggs from Naked Neck hens. The use of appropriate storage methods, such as oil coatings on the eggshell, can dramatically reduce weight loss and preserve shell thickness, thereby better-maintaining egg quality.

The internal quality of the eggs was assessed by measuring albumen height, HU, yolk color, and the pH of both albumen and yolk (Table 2). The structure of Table 2 follows the same format as Table 1, presenting results according to the effects of storage temperature, storage duration, and their interaction. The height of the thick albumen significantly decreased ($p<0.05$) during storage in both groups of eggs, with the decline being more pronounced and statistically significant ($p<0.05$) at room temperature, indicating protein structure degradation. A similar trend was observed for HU, which followed the decline in albumen height. A statistically significant difference ($p<0.05$) in HU values was found under the influence of both factors. HU values decreased during storage, with a more pronounced decline at room temperature. The results of Kanasuah et al. (2025) are consistent with our findings regarding the internal egg quality of the Naked Neck hens. Kaya et al. (2024) showed that higher storage temperatures cut both HU and albumen height, with the steepest quality loss at 30 °C. Kim et al. (2024) confirmed this trend, noting similar drops in the same measures. The study by An et al. (2023) shows that subsequent storage at low temperatures can mitigate the unfavorable effects of prior heat exposure, partially preserving HU. As a reliable indicator of albumen quality, HU tend to decrease during storage, which is supported by the results of Obianwuna et al. (2022). Lordelo et al. (2020) state that eggs from indigenous breeds can match the quality of commercial eggs and that breeding these birds supports genetic diversity and local farmers, which is crucial for sustainable agriculture and biodiversity. Similarly, Romero et al. (2024) note that indigenous breeds have competitive egg quality compared to commercial eggs.

Table 2. Changes in internal egg quality parameters as affected by storage temperature, storage duration, and their interaction

ST (°C)	SD (day)	Albumen height (mm)	Albumen pH	Haugh units	Yolk colour (Roche)	Yolk pH
		x̄±Sd	x̄±Sd	x̄±Sd	x̄±Sd	x̄±Sd
RF		5.25^{a}±1.13	8.97^{b}±0.27	71.39^{a}±9.88	13.30±0.47	6.19^{b}±0.11
RT		4.57^{b}±1.44	9.24^{a}±0.29	64.36^{b}±13.86	13.29±0.39	6.24^{a}±0.14
	1	6.54^{a}±0.75	8.63^{c}±0.35	81.35^{a}±5.53	13.00±0.60	6.08^{b}±0.07
	7	5.50^{b}±0.96	9.08^{ab}±0.13	73.51^{b}±8.51	13.08±0.51	6.11^{b}±0.06
	14	4.76^{bc}±0.70	9.12^{b}±0.14	67.64^{ab}±7.14	13.75±0.71	6.27^{a}±0.07
	21	4.24^{cd}±0.80	9.23^{a}±0.12	62.59^{cd}±8.68	13.25±0.45	6.28^{a}±0.09
	28	3.50^{cb}±0.97	9.25^{a}±0.14	54.29^{d}±11.71	13.42±0.51	6.34^{a}±0.08
RF	1	6.48±0.84	8.57±0.38	80.88±6.42	13.17±0.41	6.09±0.08
	7	5.72±1.06	9.00±0.11	75.34±8.79	13.33±0.52	6.11±0.09
	14	5.05±0.83	9.01±0.07	70.15±8.68	13.31±0.53	6.21±0.05
	21	4.80±0.70	9.12±0.06	68.06±7.50	13.32±0.51	6.24±0.11
	28	4.20±0.87	9.14±0.12	62.51±8.92	13.33±0.52	6.29±0.03
RT	1	6.60±0.72	8.69±0.35	81.81±5.05	12.83±0.75	6.06±0.06
	7	5.28±0.89	9.15±0.09	72.68±6.60	12.84±0.41	6.11±0.03
	14	4.46±0.43	9.23±0.08	65.12±4.65	14.17±0.40	6.32±0.05
	21	3.68±0.42	9.33±0.05	57.13±6.14	13.17±0.41	6.30±0.06
	28	2.80±0.35	9.37±0.04	46.07±7.75	13.50±0.55	6.38±0.11
				p value		
ST		< 0.001	<0.001	<0.001	1.000	<0.013
SD		<0.001	<0.001	<0.001	0.282	<0.001
ST x SD		0.124	0.937	0.055	0.408	0.115

ST - Storage temperature; SD - Storage duration; RF - refrigeration temperature (4–6°C); RT - room temperature (20–22°C); x̄ - Average mean; Sd - Standard deviation; p<0.05 significant; p>0.05 not significant; [a, b, c, d] Different letters indicate significant differences among the means in each column with the same storage temperature

The yolk color mainly remained stable during storage, and had a slightly higher value in eggs stored at lower temperatures. Islam et al. (2001) recorded a higher yolk colour value in Naked Neck chickens, which is in agreement with the findings of Kanasuah et al. (2025). Yolk colour is believed to be primarily influenced by diet rather than genetics (Grashorn, 2016), which could explain the inconsistent results reported in the literature. Barbosa et al. (2011) showed that the color intensity, assessed visually, remained stable during 35 days of storage in the refrigerator, while at room temperature, color reduction was observed only after 28 days. However, the same authors report that spectrophotometric analysis revealed a gradual decline in carotenoid concentration in the yolk regardless of storage temperature, indicating that the visual appearance of color may remain unchanged despite chemical changes. The stability of yolk color has also been confirmed by recent studies, with modern methods such as fluorescent spectroscopy showing potential for detecting changes in pigmentation and yolk freshness in a nondestructive manner (Atwa et al., 2024). Gu et al. (2024) highlight that fluorescent spectroscopy detects chemical changes before visible color changes, allowing for a more precise assessment of egg freshness and quality during storage.

The pH of the albumen showed a highly statistically significant ($p<0.05$) increase during storage under the influence of both temperatures, resulting from carbon dioxide loss and increased acidity of the albumen. The differences in albumen pH values between day 21 and day 28 were not statistically significant, indicating that the pH remained stable during this period. Additionally, the pH of the yolk showed statistically significant ($p<0.05$) differences depending on temperature and significant ($p<0.05$) differences due to storage duration. Over time, the pH of the yolk increased at both temperatures. The differences in yolk pH values between day 1 and day 7 were not statistically significant. However, a statistically significant increase in this parameter was observed with further storage. These results are consistent with the findings of An et al. (2023), who report that the pH of the albumen increased until the sixth day of storage and stabilized until the 15th day, depending on the storage temperature. Jin et al. (2010) also report a sharp increase in the pH of the albumen within 5 days, reaching 9.0, after which the level remained stable during further storage. Kaya et al. (2024) and Kim et al. (2024) confirm that storing eggs at higher temperatures increases the pH values of both albumen and yolk.

Microbiological quality is a key indicator of egg safety during storage and distribution. In the present study, although no pathogenic bacteria such as *Salmonella* spp. or *Listeria monocytogenes* were detected in either storage condition, variations in TBC were observed over the 28-day storage period. Table 3 summarizes the changes in total bacterial count (TBC) in eggs during storage. As with Tables 1 and 2, the results are structured to reflect the individual and combined effects of storage temperature and duration. These variations support the hypothesis that storage temperature plays a key role in bacterial proliferation, even in the absence of detectable pathogens. Our results demonstrated that eggs stored at both temperatures exhibited a gradual increase in total bacterial count, with a significant ($p<0.05$) difference observed on day 28.

These modest yet microbiologically relevant changes agreed with previous findings, which reported faster microbial proliferation at ambient temperatures and stressed that keeping eggs below 10°C inhibits bacterial growth, metabolism, and reproduction and helps preserve internal quality (Messens et al., 2006). De Reu et al. (2005) similarly found that eggshell bacterial contamination was significantly higher when eggs were kept at room temperature, particularly in systems with more exposure to environmental contaminants. Although our study handled the eggs under sterile conditions, the native shell microbiota present on the shell and in the environment could still have contributed to the overall microbial load, especially at higher temperatures. Similarly, Tan et al. (2023) found that cold chain maintenance in post-washing and storage stages contributes to reduced microbial risks and extended egg shelf life. Even though the absence of Salmonella and Listeria monocytogenes is favorable, an increase in total bacterial load at room temperature underlines the need for effective refrigeration practices. Therefore, based on our findings and consistent with literature data, it is evident that refrigerated storage remains a fundamental factor in maintaining the microbiological safety of eggs. These results further support regulatory guidelines promoting the preservation of the cold chain throughout egg handling and distribution.

Table 3. Total Bacterial Count (TBC) of eggs depending on storage temperature, storage duration, and their interaction

ST (°C)	SD (day)	Total Bacterial Count (log CFU/mL)
		$\bar{x} \pm$ Sd
RF		1.26 ± 0.30
RT		1.30 ± 0.18
	1	$1.20^{b} \pm 0.13$
	7	$1.23^{b} \pm 0.13$
	14	$1.25^{b} \pm 0.13$
	21	$1.27^{b} \pm 0.14$
	28	$1.44^{a} \pm 0.16$
RF	1	1.20 ± 0.12
	7	1.20 ± 0.11
	14	1.24 ± 0.15
	21	1.25 ± 0.12
	28	1.37 ± 0.13
RT	1	1.21 ± 0.15
	7	1.25 ± 0.16
	14	1.26 ± 0.11
	21	1.28 ± 0.17
	28	1.50 ± 0.18
		p value
	ST	0.243
	SD	0.002
	ST x SD	0.801

ST - Storage temperature; SD - Storage duration; RF - refrigeration temperature (4–6°C); RT - room temperature (20–22°C); $\bar{x}$ - Average mean; Sd - Standard deviation; $p<0.05$ significant; $p>0.05$ not significant; [a,b] Different letters indicate significant differences among the means in each column with the same storage temperature

Conclusion

During the four-week trial, temperature emerged as the dominant driver of quality change in Banat Naked Neck eggs. Storage of eggs at a lower temperature (4–6°C) significantly slowed the decline in internal quality parameters (albumen height and HU), as well as the increase in pH of the albumen and yolk, compared to storage at room temperature (20–22°C). No significant differences were observed in egg weight, shell quality characteristics, and microbiological safety of the eggs between the two storage conditions. Storage time significantly affected on the reduction of albumen height and HU, the increase in the pH of albumen and yolk, and the increase in the total bacterial count. The obtained results highlight the importance of implementing a cold chain in maintaining the quality and safety of eggs during prolonged storage.

In addition, by using eggs from Banat Naked Neck hens, a native Serbian breed, this research supports the recognition and preservation of local poultry genetic resources. Further studying the quality and storage characteristics of eggs from native breeds provides scientific evidence that can help promote their use in sustainable farming systems and in markets that value biodiversity and the authenticity of traditional products.

Uticaj temperature i dužine skladištenja na mikrobiološku ispravnost, kvalitet i održivost jaja kokoši Banatskih gološijana pre isteka roka trajanja

Maja Petričević, Maša Radojičić, Veselin Petričević, Tamara Stamenić, Tanja Keškić, Slobodan Dolašević, Zlata Kralik

Rezime

Temperatura je ključni činilac degradacije kvaliteta jaja, međutim, podaci o jajima kokoški rase Banatski gološijan i dalje su ograničeni. Ovo istraživanje obuhvatilo je 240 svežih jaja skladištenih tokom 28 dana na sobnoj temperaturi (RT, 20–22°C) i na temperaturi frižidera (RF, 4–6°C). Na 1., 7., 14., 21. i 28. dan određivani su: masa jajeta, deformacija i čvrstoća ljuske, masa i debljina ljuske, visina belanca, Haugh-ove jedinice (HU), boja žumanca, pH belanca i žumanca; sproveden je i preliminarni mikrobiološki skrining na ukupan broj bakterija, *Salmonella* spp. i *Listeria monocytogenes*. Masa jaja je ostala stabilna. Visina belanca se značajno smanjila za 35,18 % u RF, odnosno 57,58 % na RT, kada se uporede prvi i poslednji dan četvoronedeljnog perioda skladištenja, dok su se HU smanjile sa 80 na 62 u RF, odnosno, sa 81 na 46 na RT ($p<0,05$). Čvrstoća ljuske, debljina ljuske i boja žumanca ostale su statistički nepromenjene. pH belanca porastao je na 9,14 u RF i 9,37 na RT ($p<0,05$), a pH žumanca postupno je rastao prateći isti temperaturno zavisan trend ($p<0,05$). Ciljani patogeni nisu detektovani ni u jednom tretmanu. Tokom perioda skladištenja zabeleženo je postupno povećanje ukupnog broja bakterija, pri čemu je statistički veoma značajna razlika ($p<0,05$) uočena 28. dana. Rezultati ovog rada pokazuju važnost održavanja intaktnog lanca i pravilnog skladištenja u roku trajanja za očuvanje optimalnog kvaliteta i bezbednosti jaja.

Ključne reči: mikrobiološka ispravnost, kvalitet jaja, uslovi skladištenja, održivost, Banatski gološijan

Acknowledgement
The results of this research were financed by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia - SRO No. 451-03-33/2026-03/200022.

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

References

Akter Y., Kasim A., Omar H., Sazili A. Q. 2014. Effect of storage time and temperature on the quality characteristics of chicken eggs. *Journal of Food, Agriculture & Environment*, 12(3-4), 87-92.

An J. H., Hwang Y., Hwang S., Kwon H., Gu H., Park K., Choi C. 2023. Comparative Evaluation of Egg Quality in Response to Temperature Variability: From Farm to Table Exposure Scenarios. *Food Science of Animal Resources*, 43(6), 1002.

Atwa E. M., Xu S., Rashwan A. K., Abdelshafy A. M., ElMasry G., Al-Rejaie S. et al. 2024. Advances in Emerging Non-Destructive Technologies for Detecting Raw Egg Freshness: A Comprehensive Review. *Foods*, 13(22), 3563.

Barbosa V.C., Gaspar A., Calixto L.F.L., Agostinho T.S.P. 2011. Stability of the pigmentation of egg yolks enriched with omega-3 and carophyll stored at room temperature and under refrigeration. *Revista Brasileira de Zootecnia*, 40, 1540-1544.

Chousalkar K.K., Khan S., McWhorter A.R. 2021. Microbial quality, safety and storage of eggs. *Current Opinion in Food Science*, 38, 91-95.

Crivei I.C., Marc R.A., Rațu R.N., Mureșan C.C., Postolache A.N., Stoica F. et al. 2025. Advanced risk and hazard analysis in the egg sorting-packing units industry from supplier selection to delivery in chain stores under Global Food Safety Initiative integrated food safety programs. *Heliyon*, 11(7):e42587.

De Reu K., Grijspeerdt K., Heyndrickx M., Zoons J., De Baere K., Uyttendaele M. et al. 2005. Bacterial eggshell contamination in conventional cages, furnished cages and aviary housing systems for laying hens. *British Poultry Science*, 46(2), 149-155.

European Commission. 2008. Commission Regulation (EC) No. 589/2008 of 23 June 2008 laying down detailed rules for implementing Council Regulation (EC) No. 1234/2007 as regards marketing standards for eggs. *Official Journal of the European Union*, 50, 6-23.

Gogo J.A., Atitwa B.E., Gitonga C.N., Mugo D.M. 2021. Modelling conditions of storing quality commercial eggs. *Heliyon*, 7(8):e07868.

Gole V.C., Chousalkar K.K., Roberts J.R., Sexton M., May D., Tan J., Kiermeier A. 2014. Effect of egg washing and correlation between eggshell characteristics and egg penetration by various Salmonella Typhimurium strains. *PloS one*, 9(3), e90987.

Grashorn M. 2016. Feed additives for influencing chicken meat and egg yolk color. In Carle R. and Schweiggert R.M. (Eds), *Handbook on natural pigments in food and beverages* (283-302). Woodhead Publishing Series in Food Science, Technology and Nutrition.

Gu H., Hu L., Dong Y., Chen Q., Wei Z., Lv R. 2024. Evolving trends in fluorescence spectroscopy techniques for food quality and safety: A review. *Journal of Food Composition and Analysis*, 106212.

Hagan J.K., Eichie F.O. 2019. Egg quality of two layer strains as influenced by extended storage periods and storage temperatures. *Livestock Research for Rural Development*, 31(9), 1-8.

Hayder A.A.H., Aamer R.A.A. 2024. Bacterial contamination of chicken eggs: A review. *South Asian Journal of Agricultural Sciences*, 4(2), 91-93.

Holt P.S., Davies R.H., Dewulf J., Gast R.K., Huwe J.K., Jones D.R. et al. 2011. The impact of different housing systems on egg safety and quality. *Poultry Science*, 90(1), 251-262.

Hutchinson M.L., Gittins J., Walker A., Sparks N., Humphrey T.J., Burton C. et al. 2004. An assessment of the microbiological risks involved with egg washing under commercial conditions. *Journal of Food Protection*, 67(1), 4-11.

Islam M.A., Bulbul S.M., Seeland G., Islam A.B.M.M. 2001. Egg quality of different chicken genotypes in summer and winter. *Pakistan Journal of Biological Sciences*, 4(11), 1411-1414.

Institute for Standardization of Serbia. 2017. SRPS EN ISO 6887-4:2017 – Microbiology of the food chain – Preparation of test samples, initial suspension and decimal dilutions for microbiological examination – Part 4: Specific rules for the preparation of dairy products. Belgrade: Institute for Standardization of Serbia.

Institute for Standardization of Serbia. 2017. SRPS EN ISO 4833-2:2017 – Microbiology of the food chain – Horizontal method for the enumeration of microorganisms – Part 2: Colony count at 30°C by the membrane filtration technique. Belgrade: Institute for Standardization of Serbia.

Institute for Standardization of Serbia. 2017. SRPS EN ISO 6579-1:2017 – Microbiology of the food chain – Horizontal method for the detection of *Salmonella* spp. – Part 1: Detection of Salmonella spp. Belgrade: Institute for Standardization of Serbia.

Institute for Standardization of Serbia. 2017. SRPS EN ISO 11290-1:2017 – Microbiology of the food chain – Horizontal method for the detection and enumeration of *Listeria monocytogenes* and *Listeria* spp. – Part 2: Enumeration method. Belgrade: Institute for Standardization of Serbia.

Jin Y.H., Lee K.T., Lee W.I., Han Y.K. 2010. Effects of storage temperature and time on the quality of eggs from laying hens at peak production. *Asian-Australasian Journal of Animal Sciences*, 24(2), 279-284.

Jones D.R., Ward G.E., Regmi P., Karcher D.M. 2018. Impact of egg handling and conditions during extended storage on egg quality. *Poultry Science*, 97(2), 716-723.

Kanasuah D.N., Adomako K., Hagan B.A., Olympio O.S. 2025. Influence of feather genotype, storage duration and temperature on the external and internal qualities of chicken table eggs. *Online Journal of Animal and Feed Research*, 15(1):21-32.

Kaya S., Özdemir H.H., Erdem B.G. 2024. Physical changes in hen eggs stored at different temperatures. *Food and Health*, 10(4), 253-261.

Kim Y.B., Lee S.Y., Yum K.H., Lee W.T., Park S.H., Lim Y.H. et al. 2024. Effects of storage temperature and egg washing on egg quality and physicochemical properties. *Discover Applied Sciences*, 6(3), 111.

Kumari A., Tripathi U.K., Maurya V., Kumar M. 2020. Internal quality changes in eggs during storage. *International Journal of Science, Environment and Technology*, 9(4), 615-624.

Lordelo M., Cid J., Cordovil C.M., Alves S.P., Bessa R.J., Carolino I. 2020. A comparison between the quality of eggs from indigenous chicken breeds and that from commercial layers. *Poultry Science*, 99(3), 1768-1776.

Mahmoud B.Y., Semida D.A., Elnesr S.S., Elwan H., El-Full E.A. 2022. Approaches of egg decontamination for sustainable food safety. *Sustainability*, 15(1), 464.

Messens W., Grijspeerdt K., Herman L. 2006. Eggshell penetration of hen's eggs by Salmonella enterica serovar Enteritidis upon various storage conditions. *British Poultry Science*, 47(5), 554-560.

Nowaczewski S., Babuszkiewicz M., Szablewski T., Stuper-Szablewska K., Cegielska-Radziejewska R., Kaczmarek S. et al. 2022. Effect of weight and storage time of broiler breeders' eggs on morphology and biochemical features of eggs, embryogenesis, hatchability, and chick quality. A*nimal*, 16(7), 100564.

Obianwuna U.E., Oleforuh-Okoleh V.U., Wang J., Zhang H.J., Qi G.H., Qiu K. et al. 2022. Potential implications of natural antioxidants of plant origin on oxidative stability of chicken albumen during storage: A review. *Antioxidants*, 11(4), 630.

Razi S.M., Fahim H., Amirabadi S., Rashidinejad A. 2023. An overview of the functional properties of egg white proteins and their application in the food industry. *Food Hydrocolloids*, 135, 108183.

Rho T.G., Cho B.K. 2024. Non-Destructive Evaluation of Physicochemical Properties for Egg Freshness: A Review. *Agriculture*, 14(11), 2049.

Romero C., Yustos J.L., Sánchez-Román I., López-Torres M., Chamorro S. 2024. Assessment of performance and egg quality in laying hens of Spanish indigenous breed Black Castellana as compared with a selected white egg-layer strain. *Poultry Science*, 103(10), 104096.

Serbian Regulation. 2019a. *Regulation on egg quality* (Nos. 7/2019-3, 35/2019-54, 78/2019-18). *Official Gazette of the Republic of Serbia*. Belgrade, Serbia.

Škrbić Z., Lukić M., Petričević V., Bogosavljević-Bošković S., Rakonjac S., Dosković V., Tolimir N. 2020. Quality of eggs from pasture rearing layers of different genotypes. *Biotechnology in Animal Husbandry*, 36(2), 181-190.

Škrbić Z., Lukić M., Petričević V., Bogosavljević-Bošković S., Rakonjac S., Dosković V., Tolimir N. 2021. Egg quality of Banat naked neck hens during storage. *Biotechnology in Animal Husbandry*, 37(2), 87-97.

Solís D., Cordero N., Quezada-Reyes M., Escobar-Astete C., Toro M., Navarrete P., Reyes-Jara A. 2023. Prevalence of Salmonella in Eggs from Conventional and Cage-Free Egg Production Systems and the Role of Consumers in Reducing Household Contamination. *Foods*, 12(23), 4300.

Statistical Office of the Republic of Serbia. 2025. Food production and consumption in Serbia. Retrieved June 2, 2025, from https://www.stat.gov.rs

Tan F.J., Simsiri U., Sun Y.M. 2023. Effect of Cold Chain on Chicken Egg Quality in a Simulated Post Washing Processing and Consumer Storage Model. *Brazilian Journal of Poultry Science*, 25(03), eRBCA-2021.

The Business Research Company. 2024. Egg Global Market Report 2024. https://www.einpresswire.com/article/696182228/egg-market-size-share-and-growth-analysis-for-2024-2033

Wellman-Labadie O., Picman J., Hincke M.T. 2008. Antimicrobial activity of the Anseriform outer eggshell and cuticle. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 149(4), 640-649.

Wengerska K., Batkowska J., Drabik K. 2023. The eggshell defect as a factor affecting the egg quality after storage. *Poultry Science*, 102(7), 102749.

Yamak U.S., Sarica M., Erensoy K., Ayhan V. 2021. The effects of storage conditions on quality changes of table eggs. *Journal of Consumer Protection and Food Safety*, 16(1), 71-81.

Zhi X., Xing Y., Huang D., Tong Q., Du B., Wu J. 2016. Influences of Cleaning and Coating Conditions on the Quality of Eggs. In *Advanced Graphic Communications, Packaging Technology and Materials* (pp. 541-547). Springer Singapore.

Received 15 July 2025; accepted for publication 26 January 2026

Biotechnology in Animal Husbandry 42(1), 77-86, 2026
Publisher: Institute for Animal Husbandry, Belgrade-Zemun

ISSN 1450-9156
UDC 619'636.2
https://doi.org/10.2298/BAH2601077D

RELATIONSHIP BETWEEN SERUM ZINC CONCENTRATION, TEAT CANAL KERATIN THICKNESS AND SOMATIC CELL COUNT IN DAIRY COWS

Ivana Davidov, Aleksandar Božić, Annamaria Galfi Vukomanović, Ksenija Čobanović, Nikola Davidov, Bojana Blagojević, Željka Jurakić

University of Novi Sad, Faculty of Agriculture, Novi Sad, Serbia
Corresponding author: Ivana Davidov, ivana.davidov@polj.edu.rs
Original scientific paper

Abstract: The aim of this study was to evaluate the relationship between serum zinc concentration, teat canal keratin layer thickness and somatic cell count (SCC) in dairy cows. The study included 26 dairy cows excluded from production and sent to slaughter, with a total of 104 udder quarters examined. Prior to slaughter, blood samples were collected for determination of serum zinc concentration and milk samples from each udder quarter were obtained for SCC analysis. After slaughter, teats were collected for histopathological examination and the thickness of the teat canal keratin layer was measured. A significant negative correlation was observed between serum zinc concentration and SCC ($r = –0.62$, $p < 0.01$), indicating that higher serum zinc levels were associated with lower SCC values. Serum zinc concentration was positively correlated with teat canal keratin layer thickness ($r = 0.58$, $p < 0.01$), while keratin layer thickness showed a significant negative relationship with SCC ($r = –0.54$, $p < 0.05$). The lowest SCC values were recorded in teat canals with a keratin layer thickness of 201–300 µm, whereas higher SCC values were found in canals with both very thin (0–100 µm) and excessively thick (301–400 µm) keratin layers. These results indicate that adequate serum zinc concentration and optimal teat canal keratin layer thickness are associated with improved udder health and lower SCC values, suggesting a potential role of zinc status and teat canal integrity in reducing the risk of subclinical mastitis in dairy cows.

Key words: dairy cows, zinc, teat canal, keratin layer, somatic cell count

Introduction

Mastitis remains one of the most significant health problems in dairy cattle, causing substantial economic losses due to reduced milk yield, impaired milk quality, increased veterinary costs and higher culling rates (Ranasinghe et al., 2021; Shafeeq et al., 2021; Stanek et al., 2024; Yu et al., 2025). Subclinical mastitis, characterized by the absence of visible clinical signs, represents a particular challenge, as it often remains undetected while negatively affecting milk production and milk quality (Hamadani et al., 2013; Cobirka et al., 2020). Somatic cell count (SCC) is widely used as a reliable indicator of udder health and the presence of subclinical intramammary infections.

The teat canal constitutes the first line of defense against the penetration of pathogenic microorganisms into the mammary gland. Its protective function is largely attributed to the keratin layer lining the teat canal, which provides both a physical barrier and bacteriostatic properties (Ezzat Alnakip et al., 2014; Cardozo et al., 2025). Structural alterations of the teat canal keratin, including thinning, excessive keratinization or irregular keratin arrangement, may impair its barrier function and facilitate bacterial invasion, leading to increased SCC and a higher risk of intramammary infection (Capuco et al., 1992; Pantoja et al., 2020).

Zinc is an essential trace element involved in numerous biological processes, including epithelial cell proliferation, differentiation and keratinization. Adequate zinc status is crucial for maintaining the integrity of epithelial and mucosal barriers, as well as for optimal immune function. Zinc deficiency has been associated with impaired keratin formation, delayed epithelial regeneration and increased susceptibility to infectious diseases (Hassan et al., 2020; Zemanova et al., 2022). Several studies have demonstrated the beneficial effects of zinc supplementation on udder health and somatic cell count in dairy cows (Pechová et al., 2006; Davidov et al., 2014; Oconitrillo et al., 2024; Li et al., 2025). But however, most available data focus either on systemic zinc status or on production parameters, while limited information is available on the relationship between serum zinc concentration, teat canal keratin morphology and SCC assessed simultaneously at the level of individual udder quarters.

Therefore, the present study was designed to test the hypothesis that serum zinc concentration is associated with the thickness of the teat canal keratin layer and that both parameters are related to somatic cell count in dairy cows. Specifically, the aim of this study was to investigate the relationship between serum zinc concentration, teat canal keratin layer thickness and SCC in dairy cows, with the objective of contributing to a better understanding of the role of zinc status and teat canal integrity in udder health and the prevention of subclinical mastitis.

Materials and Methods

The study was conducted on 26 dairy cows that were excluded from production and sent for slaughter. A total of 104 udder quarters were included in the analysis. Prior to slaughter, blood samples were collected from the coccygeal vein, and milk samples were collected separately from each udder quarter for SCC analysis. As all samples were obtained from animals already designated for commercial slaughter, and procedures were limited to routine blood and milk collection that did not interfere with the slaughter process or cause additional animal distress, formal approval from an ethics committee was not required according to national regulations.

Blood samples were obtained from the caudal vein, following standard principles of asepsis and antisepsis while minimizing animal distress. Samples were collected into additive-free vacutainer tubes (BD Vacutainer Systems, Preanalytical Solutions, UK) to obtain serum. Each vacutainer was labeled with the cow identification number and kept at room temperature until further processing.

Blood samples were centrifuged to obtain serum, which was stored under appropriate conditions until analysis. Serum zinc concentration was determined by flame atomic absorption spectrophotometry using a (PerkinElmer PinAAcle 900) spectrometer. To minimize matrix interference, serum samples were diluted 1:5 with 0.1 M deionized nitric acid (HNO). The analysis was performed at a wavelength of 213.9 nm, using a zinc hollow cathode lamp as the radiation source and an air-acetylene flame. A standard calibration curve (0.1–2.0 mg/L) was prepared using a certified reference zinc solution. The results were expressed in µmol/L.

Milk samples were collected from all four udder quarters. Prior to sampling, teat ends were thoroughly disinfected. Each milk sample bottle was labeled with the cow identification number. Somatic cell count was determined using the fluoro-opto-electronic method with a Fossomatic analyzer (Foss Electric, Hillerød, Denmark). Subclinical mastitis was defined as an SCC exceeding 400×10^3 cells/mL (Pyörälä, 2003).

After slaughter, teats were collected from each udder quarter. Tissue samples were fixed in 10% neutral buffered formalin for 48 h, routinely processed, and embedded in paraffin. Sections of 5 µm thickness were cut and stained with hematoxylin and eosin (H&E) according to the methods described by Senthilkumar et al. (2020). Morphometric analysis of the teat canal was performed using a light microscope (Olympus BX51) equipped with a digital camera. The thickness of the keratin layer was measured at five random points per section using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Prior to measurement, the software was calibrated using a stage micrometer to convert pixels into micrometers (µm).

Statistical analysis was performed using IBM SPSS Statistics for Windows, version 25.0 (IBM Corp., Armonk, NY, USA). Descriptive statistics, including mean, standard deviation, and frequency distributions, were calculated for all examined parameters. The normality of the data distribution was assessed using the Shapiro-Wilk test. Differences in SCC and zinc levels among teat canals grouped by keratin layer thickness were analyzed using one-way analysis of variance (ANOVA). Furthermore, the strength and direction of the relationships between serum zinc, keratin layer thickness, and SCC were evaluated using Pearson's correlation coefficient (r). Statistical significance was set at $p < 0.05$ and $p < 0.01$. Due to the hierarchical structure of the data (multiple quarters per cow), results were interpreted with caution regarding the independence of observations.

Results and Discussion

The results of the present study indicate a clear association between serum zinc concentration, keratin layer thickness of the teat canal and somatic cell count (SCC), suggesting an important role of zinc status and teat canal integrity in udder health.

The distribution of serum zinc concentrations and SCC values among the examined cows is shown in Table 1.

Table 1. Descriptive statistics of serum zinc concentration and SCC in the examined cows (n=26)

Parameter	Zn conc. (μmol/L)	SCC x10^3/mL
Cow 1	11.66	440
Cow 2	13.62	450
Cow 3	11.79	440
Cow 4	20.47	290
Cow 5	12.56	400
Cow 6	19.28	310
Cow 7	14.07	390
Cow 8	13.28	490
Cow 9	6.04	460
Cow 10	1.75	420
Cow 11	3.12	470
Cow 12	7.88	400
Cow 13	4.56	430
Cow 14	15.07	410
Cow 15	16.41	390
Cow 16	22.64	310
Cow 17	15.76	410
Cow 18	15.05	450
Cow 19	16.76	430

Table 1. continue

Parameter	Zn conc. (µmol/L)	SCC x10^3/mL
Cow 20	16.08	440
Cow 21	20.35	290
Cow 22	17.51	310
Cow 23	13.23	470
Cow 24	18.12	380
Cow 25	18.22	350
Cow 26	17.59	370
Mean ± SD	13.91 ± 5.37	398.08 ± 56.50
Median	15.06	410
Minimum	1.75	490
Maximum	22.64	290
Range	20.89	200

Considerable individual variability in serum zinc concentration and SCC was observed among the examined cows (Table 1), which is consistent with previous studies reporting differences in mineral status and susceptibility to subclinical mastitis in dairy cattle (Davidov et al., 2012; Li et al., 2025). Cows with higher serum zinc concentrations generally exhibited lower SCC values.

Histopathological examination of 104 teat canals showed substantial variability in keratin layer thickness. The highest proportion of teat canals exhibited a keratin layer thickness of 201–300 µm (37.50%), followed by canals with a thickness of 301–400 µm (32.69%). Thinner keratin layers (≤200 µm) were observed less frequently. Such variability in keratin layer thickness has also been reported by other authors and may reflect differences in teat canal exposure, milking conditions and epithelial turnover (Pantoja et al., 2020).

Differences in keratin layer thickness were associated with variations in somatic cell count (Table 2). The lowest mean SCC (339 × 10^3 cells/mL) was observed in teat canals with a keratin layer thickness of 201–300 µm. Slightly higher SCC values were recorded in canals with a thickness of 101–200 µm (408 × 10^3 cells/mL). In contrast, markedly higher SCC values were detected in teat canals with very thin keratin layers (0–100 µm; 465 × 10^3 cells/mL) as well as in canals with excessive keratinization (301–400 µm; 424 × 10^3 cells/mL). These findings suggest that both insufficient and excessive keratin layer thickness may compromise the protective function of the teat canal and increase susceptibility to subclinical mastitis (Pantoja et al., 2020; Cardozo et al., 2025).

These findings suggest that not only an underdeveloped but also an excessively thick keratin layer may compromise the protective function of the teat canal. Excessive keratinization may lead to irregular keratin structure and fissures, potentially favoring bacterial colonization (Cardozo et al., 2025), while a thin keratin layer may provide insufficient mechanical and antimicrobial protection (Paduch et al., 2012).

Table 2. Serum zinc concentration, keratin layer thickness and SCC

No. of teat canals	Mean Zn conc. (μmol/L)	Keratin layer thickness (μm)	Mean SCC x10^3/mL
8	10.72	0-100	465
23	11.65	101-200	408
39	18.47	201-300	339
34	11.26	301-400	424

The results of this research indicate that optimal serum zinc concentration and adequate keratin layer thickness are associated with improved udder health, as reflected by lower SCC values. In our study, the mean serum zinc concentration was 13.91 ± 5.37 μmol/L. This is in agreement with Zadnik (2010), who reported that cows with healthy udders typically maintain serum zinc levels within the physiological range of 12–18 μmol/L. Furthermore, our observation of a strong negative correlation between zinc and SCC (r = -0.62) supports the findings of Zadnik (2010), who established that serum zinc concentrations below 9.64 μmol/L are significantly associated with increased SCC and a higher prevalence of subclinical mastitis. These findings underline the importance of appropriate zinc nutrition and the structural integrity of the teat canal in the prevention of intramammary infections (Davidov et al., 2013; Nudda et al., 2023).

Correlation analysis revealed a significant negative relationship between serum zinc concentration and somatic cell count (SCC) ($r = –0.62$, $p < 0.01$), indicating that higher serum zinc levels were associated with lower SCC values. A significant positive correlation was observed between serum zinc concentration and keratin layer thickness of the teat canal ($r = 0.58$, $p < 0.01$). In addition, keratin layer thickness was negatively correlated with SCC ($r = –0.54$, $p < 0.05$), suggesting a protective role of an adequately developed keratin layer against increased somatic cell counts (Table 3).

Table 3. Relationship between serum zinc concentration, keratin layer thickness and SCC

Compared variables	Correlation coefficient (r)	p-value
Serum Zn – SCC	-0.62	< 0.01
Serum Zn – keratin layer thickness	0.58	< 0.01
Keratin layer thickness – SCC	-0.54	< 0.05

The observed inverse relationship between serum zinc and SCC ($r = -0.62$; $p < 0.01$) reinforces the critical role of this microelement in udder defense mechanisms. By promoting keratin synthesis and cellular regeneration, zinc maintains the structural integrity of the teat canal, which serves as the primary barrier against bacterial entry (Zemanova et al., 2022). Consequently, the severe zinc deficiency recorded in some of our subjects (minimum 1.75 µmol/L) likely impaired this barrier function, explaining the higher inflammatory responses and SCC levels observed (Prasad, 2008; Zadnik, 2010).

In the present study, serum zinc concentration was positively correlated with the thickness of the keratin layer of the teat canal. This relationship suggests that adequate zinc status may contribute to the development and maintenance of an optimal keratin layer, which represents the first physical and chemical barrier against pathogen entry (Nickerson, 1985). The observed negative correlation between keratin layer thickness and SCC further emphasizes the protective role of the teat canal keratin in preventing inflammatory processes within the udder (Zigo et al., 2021).

Conclusion

This study demonstrates a significant relationship between serum zinc concentration, teat canal keratin layer thickness, and somatic cell count (SCC) in dairy cows. Higher serum zinc concentrations were associated with increased keratin layer thickness and lower SCC values, indicating improved udder health. Teat canals with an intermediate keratin layer thickness (201–300 µm) exhibited the lowest SCC, while both insufficient and excessive keratinization were linked to higher SCC. These findings suggest that maintaining adequate zinc status and optimal keratin layer development may contribute to the prevention of subclinical mastitis in dairy cows.

Veza između koncentracije cinka u serumu, debljine keratinskog sloja sisnog kanala i broja somatskih ćelija kod mlečnih krava

Ivana Davidov, Aleksandar Božić, Annamaria Galfi Vukomanović, Ksenija Čobanović, Nikola Davidov, Bojana Blagojević, Željka Jurakić

Rezime

Cilj ovog istraživanja je bio da se ispita odnos između koncentracije cinka u serumu, debljine keratinskog sloja kanala sise i broja somatskih ćelija (SCC) kod mlečnih krava. Istraživanje je obuhvatilo 26 mlečnih krava isključenih iz proizvodnje (iz različitih razloga) i upućenih na klanje, pri čemu su analizirane ukupno 104 četvrti vimena. Pre klanja uzimani su uzorci krvi radi određivanja koncentracije cinka u serumu, dok su uzorci mleka iz svake četvrti vimena korišćeni za analizu SCC. Nakon klanja, sise su prikupljene za patohistološko ispitivanje, a merena je debljina keratinskog sloja kanala sise. Utvrđena je značajna negativna korelacija između koncentracije cinka u serumu i SCC ($r = -0{,}62$; $p < 0{,}01$), što ukazuje na to da su više vrednosti cinka u serumu povezane sa nižim vrednostima SCC. Koncentracija cinka u serumu bila je u pozitivnoj korelaciji sa debljinom keratinskog sloja kanala sise ($r = 0{,}58$; $p < 0{,}01$), dok je debljina keratinskog sloja pokazala značajnu negativnu povezanost sa SCC ($r = -0{,}54$; $p < 0{,}05$). Najniže vrednosti SCC zabeležene su u kanalima sisa sa debljinom keratinskog sloja od 201–300 μm, dok su više vrednosti SCC utvrđene u kanalima sa veoma tankim (0–100 μm) i prekomerno debelim (301–400 μm) keratinskim slojem. Ovi rezultati ukazuju na to da su adekvatna koncentracija cinka u serumu i optimalna debljina keratinskog sloja kanala sise povezane sa boljim zdravljem vimena i nižim vrednostima SCC, što ukazuje na potencijalnu ulogu statusa cinka i integriteta kanala sise u smanjenju rizika od subkliničkog mastitisa kod mlečnih krava.

Ključne reči: mlečne krave, cink, sisni kanal, keratinski sloj, somatske ćelije

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

References

Capuco A.V., Bright S.A., Pankey J.W., Wood D.L., Miller R.H., Bitman J. 1992. Increased susceptibility to intramammary infection following removal of teat canal keratin. *Journal of Dairy Science*, 75(8), 2126-2130.

Cardozo L.L., Knob D.A., Santos P.T.D., Pelizza A., Mori A.P., Camera M., Ferraz S.M., de Assis M.Z., Thaler Neto A. 2025. Relationship between Hyperkeratosis, Teat Conformation Traits, Microbiological Isolation, and Somatic Cell Count in Milk from Dairy Cows. *Dairy*, 6(4), 45.

Cobirka M., Tancin V., Slama P. 2020. Epidemiology and Classification of Mastitis. *Animals (Basel)*, 10(12), 2212.

Davidov I., Radinović M., Erdeljan M., Jurakić Ž., Kovačević Z. 2014. Zinc Effect on Milk Somatic Cell Count in Dairy Cows. *Acta Scientiae Veterinariae*, 42, 1226.

Davidov I., Radinović M., Erdeljan M., Kovačević Z., Jurakić Ž., Radinović M.Ž. 2013. The influence of blood and milk serum zinc concentration on milk somatic cell count in dairy cows. *Biotechnology in Animal Husbandry*, 29(4), 659-664.

Davidov I., Radinović M., Erdeljan M., Stančić I., Stojanović D., Milanov D. 2012. Relationship between somatic cell count and zinc blood serum concentration in dairy cows. *Archives of Veterinary Medicine*, 5(1), 59–68.

Ezzat Alnakip M., Quintela-Baluja M., Böhme K., Fernández-No I., Caamaño-Antelo S., Calo-Mata P., Barros-Velázquez J. 2014. The Immunology of Mammary Gland of Dairy Ruminants between Healthy and Inflammatory Conditions. *Journal of Veterinary Medicine*, 2014, 659801.

Hamadani H., Khan A.A., Banday M.T., Ashraf I., Handoo N., Bashir A., Hamadani A. 2013. Bovine Mastitis - A Disease of Serious Concern for Dairy Farmers. *International Journal of Livestock Research*, 3(1), 42-55.

Hassan A., Sada K.K., Ketheeswaran S., Dubey A.K., Bhat M.S. 2020. Role of Zinc in Mucosal Health and Disease: A Review of Physiological, Biochemical, and Molecular Processes. *Cureus*, 12(5), e8197.

Illek J., Dresler S., Šoch M., Kernerová N., Líbalová K.Š., Zevlová E., Havrdová N. 2023. Influence of organic zinc on lactation performance and somatic cell count in dairy cows. *Acta Veterinaria Brno*, 92, 233-241.

Li X., Wang J., Jiang M., Huo Y., Zhan K. 2025. Effects of Zinc (Zn) from Different Sources on Production Performance, Health Status, Antioxidant Properties and Immune Regulation of Dairy Cows in Early Lactation. *Veterinary Sciences*, 12(6), 545.

Nickerson S.C. 1985. Immune mechanisms of the bovine udder: an overview. *Journal of American Veterinary Medicine Association*, 187(1), 41-45.

Nudda A., Carta S., Battacone G., Pulina G. 2023. Feeding and Nutritional Factors That Affect Somatic Cell Counts in Milk of Sheep and Goats. *Veterinary Sciences*, 10(7), 454.

Oconitrillo M., Wickramasinghe J., Omale S., Beitz D., Appuhamy R. 2024. Effects of Elevating Zinc Supplementation on the Health and Production Parameters of High-Producing Dairy Cows. *Animals (Basel)*, 14(3), 395.

Pantoja J.C.F., Correia L.B.N., Rossi R.S., Latosinski G.S. 2020. Association between teat-end hyperkeratosis and mastitis in dairy cows: A systematic review. *Journal of Dairy Science*, 103(2), 1843-1855.

Paduch J.H., Mohr E., Krömker V. 2012. The association between teat end hyperkeratosis and teat canal microbial load in lactating dairy cattle. *Veterinary Microbiology*, 158(3-4), 353-359.

Pechová A., Pavlata L., Lokajová E. 2006. Zinc Supplementation and Somatic Cell Count in Milk of Dairy Cows. *Acta Veterinaria Brno*, 75, 355-361.

Prasad A.S. 2008. Zinc in human health: effect of zinc on immune cells. *Molecular Medicine*, 14(5-6), 353-357.

Pyörälä S. 2003. Indicators of inflammation in the diagnosis of mastitis. *Veterinary Research*, 34(5), 565-578.

Ranasinghe R.M.S.B.K., Deshapriya R.M.C., Abeygunawardana D.I., Rahularaj R. Dematawewa C.M.B. 2021. Subclinical mastitis in dairy cows in major milk-producing areas of Sri Lanka: Prevalence, associated risk factors, and effects on reproduction. *Journal of Dairy Science*, 104, 12900–12911.
Senthilkumar P., Geetha R., Parthiban M., Kumar S.S. 2020. Histomorphological evaluation of teat of Sahiwal and Holstein Friesian cattle during lactating and non-lactating periods. *Indian Journal of Animal Sciences*, 90(11), 1475-1479.
Shafeeq M., Muneer A., Aqib A.I., Kirn N. 2021. Economic Impacts of Clinical and Sub Clinical Mastitis on Dairy Farms. *Veterinary Science Research*, 3(2), 31-39.
Stanek P., Żółkiewski P., Januś E. 2024. A Review on Mastitis in Dairy Cows Research: Current Status and Future Perspectives. *Agriculture*, 14(8), 1292.
Yu W., Zhang Z., Wang Z., Lin X., Dong X., Hou Q. 2025. Comprehensive Prevention and Control of Mastitis in Dairy Cows: From Etiology to Prevention. *Veterinary Sciences*, 12(9), 800.
Zadnik T. 2010. Relations between blood zinc concentrations and udder health in dairy cows. *Veterinary Medicine and Zootechnics*, 52(74), 79-83.
Zemanova M., Langova L., Novotná I., Dvorakova P., Vrtkova I., Havlicek Z. 2022. Immune mechanisms, resistance genes, and their roles in the prevention of mastitis in dairy cows. *Archives Animal Breeding*, 65(4), 371-384.
Zigo F., Vasil M., Ondrašovičová S., Výrostková J., Bujok J., Pecka-Kielb E. 2021. Maintaining Optimal Mammary Gland Health and Prevention of Mastitis. *Frontiers in Veterinary Science*, 8, 607311.

Received 2 February 2026; accepted for publication 4 May 2026

Biotechnology in Animal Husbandry 42(1), 87-98, 2026
Publisher: Institute for Animal Husbandry, Belgrade-Zemun

ISSN 1450-9156
UDC 636.083.31'637.05
https://doi.org/10.2298/BAH2601087I

PRE-MORTEM SOUND LEVEL MEASUREMENT: NON-INVASIVE TOOL FOR WELFARE AND MEAT QUALITY ASSESSMENT IN PIGS

Mina Ilić[1], Branko Suvajdžić[1], Đorđe Pajičić[2], Milena Radaković[3], Ružica Trailović[4], Nikola Čobanović[1]

[1] Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, University of Belgrade, Bulevar oslobodjenja 18, 11000 Belgrade, Serbia
[2] Veterinary Specialised Institute "Sombor", Staparski put 35, 25000 Sombor, Serbia
[3] Department of Pathophysiology, Faculty of Veterinary Medicine, University of Belgrade, Bulevar oslobodjenja 18, 11000 Belgrade, Serbia
[4] Department of Animal Breeding, Faculty of Veterinary Medicine, University of Belgrade, Bulevar oslobodjenja 18, 11000 Belgrade, Serbia
Corresponding author: Nikola Čobanović, cobanovic.nikola@vet.bg.ac.rs
Original scientific paper

Abstract: The aim of this study was to investigate the effect of noise levels in the stunning box on oxidative stress biomarkers and meat quality traits in slaughter pigs. The experiment included 60 pigs, approximately six months old, with an average live weight of around 110 kg. For each animal, a comprehensive set of oxidative stress parameters was measured (glucose, AOPP, ceruloplasmin, GSH, TAC, TOS and the oxidative stress index), along with meat quality indicators (pH and temperature, sensory and instrumental colour, water-holding capacity and pork quality classes). Exposure of slaughter pigs to noise levels above 105 dB resulted in increased plasma TOS concentrations and the oxidative stress index. Furthermore, elevated noise levels adversely affected meat pH and temperature in the *musculus longissimus lumborum* 45 minutes post-mortem, cooking loss, *L**, *a** and *b** values, sensory colour score and the incidence of red, firm, non-exudative and pale, firm and non-exudative pork. In conclusion, the findings of this study indicate that monitoring noise levels in the stunning environment may represent a useful approach for assessing pig welfare and for predicting variations in pork quality.

Key words: noise level, oxidative stress, pig welfare, pork quality

Introduction

The meat industry and consumers are paying increasing attention to the production conditions of food, focusing on economic efficiency, animal welfare, and meat safety and quality (Sanchez et al., 2022; Svoboda et al., 2024). Conventional methods for evaluating well-being (behavioural observations, physiometabolic indicators and resource-based parameters) and meat quality (sensory evaluation, chemical composition, and technological traits) are often invasive, time-consuming, costly, and prone to errors, limiting their suitability for industrial application (Sanchez et al., 2022; Svoboda et al., 2024). Accordingly, there is a need for new, enhanced, non-invasive, rapid and reliable approaches that can be applied under practical industry conditions (Sanchez et al., 2022; Svoboda et al., 2024).

Previous studies have indicated that environmental noise intensity can act as a potential stressor for pigs (Grandin, 1996). Talling et al. (1996) reported increased heart rate and irregular behaviour in pigs when environmental noise level exceeded 85 dB. Furthermore, it has been shown that noise levels above 85 dB during the pre-slaughter period induce intense stress and negatively affect pig welfare and meat quality, leading to a higher incidence of pale, soft and exudative (PSE) meat (Vermeulen et al., 2015, 2016). Although noise measurement is considered a promising alternative approach for assessing welfare and indirectly predicting meat quality, previous studies (Vermeulen et al., 2015, 2016) have only investigated its effect on meat pH measured 30 minutes post mortem. To date, no studies have examined the impact of noise level on oxidative stress biomarkers in slaughter pigs.

Considering the aforementioned facts, the aim of this study was to investigate the impact of noise levels in the stunning box on oxidative stress biomarkers and meat quality traits in slaughter pigs.

Materials and Methods

Ethical approval

All procedures involving animals were conducted in accordance with institutional guidelines and were approved by the Local Ethics Committee for Animal Experimentation of the Faculty of Veterinary Medicine, University of Belgrade, Serbia (Approval No: 01-159 of 14 February 2025).

Experimental pigs and pre- and post-slaughter conditions

The experiment included 60 slaughter pigs (approximately 110 kg live weight and around six months old), all of uniform genetic background ([Yorkshire × Landrace] sows sired with Pietrain boars). Animals were divided into three groups (Group 1 = 12 pigs, Group 2 = 18 pigs, Group 3 = 30 pigs). The pigs originated from two commercial production units (Farm A supplied Groups 1 and 2, while Group 3 came from Farm B), where they were kept under comparable housing and management conditions. All animals underwent identical pre-slaughter handling, including loading, transport, unloading and lairage. Slaughter and carcass processing took place at two commercial abattoirs, following standard industry procedures.

Measurement of noise levels in the stunning box

A sound level meter (Testo 815, Testo GmbH, Lenzkirch, Germany) was positioned near the pig stunning box to record ambient noise. The device was set to the Fast 125 ms response mode and A-weighted decibels [dB(A)], which approximates human auditory perception. The device was placed as close as possible to the group of pigs being observed (no more than 2 m), with the microphone directed toward the primary noise source. Measurements began when pigs entered the stunning box, and peak noise values were recorded every 15 minutes, with an accuracy of ± 1.0 dB. The total duration of noise monitoring depended on the daily slaughter throughput and line speed, and measurements covered the entire stunning period.

Determination of blood glucose and oxidative stress biomarkers

Blood glucose concentration at exsanguination was determined directly on the slaughter line, within one minute of bleeding, using a portable glucometer (Accu-Chek® Performa, Roche Diagnostics, Mannheim, Germany). For the evaluation of oxidative stress biomarkers, whole-blood samples were collected during bleeding into 10 mL EDTA vacutainers and immediately placed in an insulated container with ice packs, maintaining a temperature of 3±1ºC. Upon arrival at the laboratory (approximately 180 minutes post-collection), the samples were centrifuged at 3,000 rpm for 10 minutes to obtain plasma, which was aliquoted into pre-labelled tubes and stored at -80ºC until further analysis.

Plasma ceruloplasmin activity was quantified using its p-phenylenediamine (PPD) oxidase activity, following the procedure described by Hussein et al. (2019). Reduced glutathione (GSH) levels were measured according to a modified Ellman assay (Jollow et al., 1974) using 5,5’-dithio-bis-(2-nitobenzoic acid) (DTNB). Protein oxidation was assessed by determining advanced oxidation protein products (AOPP) following the method of Witko-Sarsat et al. (1996). Total antioxidant capacity (TAC) was evaluated using the ABTS-based method described by Erel (2004), while total oxidative status (TOS) was quantified according to Erel (2005), which measured the oxidation of a ferrous ion-o-dianisidine complex by plasma oxidants. The oxidative stress index (OSI) was calculated as the TOS/TAC ratio.

Determination of pork quality traits

Pork quality assessments were carried out on the left side of each carcass. Measurement of meat pH and temperature were taken in the cooling chamber at 45 minutes and 24 hours post-slaughter using a portable pH meter (Testo 205, Testo AG, Lenzkirch, Germany; accuracy ±0.01). The probe was inserted into the *musculus longissimus lumborum* (between the 3rd and 5th lumbar vertebrae) and the central portion of the *musculus semimembranosus.*

At 24 hours post-mortem, meat samples approximately 2.5 cm thick were excised from the longissimus lumborum muscle (between 3rd and 5th vertebrae of lumbar region) for the evaluation of color and water-holding capacity traits. Subjective color scoring was performed independently by three trained evaluators using the National Pork Producers Council (2000) scale (1 = pale, 6 = dark). Objective color parameters (L^*, a^*, b^*) were determined according to the CIELab system (1996) with portable colorimeter (NR110, 3NH Technology Co., Ltd, Shenzhen, China) fitted with a 4-mm aperture, 2º observer angle and D65 illumination. Nine measurements were recorded at randomly selected sites on both the medial and lateral surfaces of the longissimus lumborum muscle to obtain representative mean values (Hunt et al., 1991).

Water-holding capacity was quanitified using three established methods: drip loss, thawing loss and cooking loss, following the procedures described by Honikel (1998) and Klauke et al. (2013).

Classification of pork quality was based on ultimate pH, differences in drip loss and L^* values, applying the scheme proposed by Correa et al. (2007). According to these criteria, samples were assigned to one of the following catagories: pale, soft and exudative (PSE); moderate PSE; pale, firm and non-exudative (PFN); red, soft and exudative (RSE); red, firm and non-exudative (RFN); moderately dark, firm and dry (moderate DFD); or dark, firm and dry (DFD).

Statistical analysis

All statistical procedures were carried out using GraphPad Prism (version 9.5.1; GraphPad Software, San Diego, CA, USA). Prior to performing the statistical analyses, normality of residuals (Shapiro–Wilk test) were evaluated for all dependent variables. The data satisfied all the required assumptions for subsequent analysis. Based on the noise levels recorded in the stunning box, pigs were classified into two groups: (1) lower noise – animals exposed to noise levels ≤105 dB (n=12); and (2) higher noise – animals exposed to noise levels ≥105 dB (n=48). Differences between the two groups were analyzed using an unpaired two-tailed t-test with Welch's correction to account for unequal variances and sample sizes. Descriptive statistics (mean and standard deviation) were used to summarize the data. Fisher's exact test was applied to evaluate significant differences in the distribution of pork quality classes. Relationships between variables were examined using Pearson's correlation coefficient (r_p). The strength of the correlation was categorized as follows: weak ($|r_p|<0.35$), moderate ($0.36\leq|r_p|<0.67$), and strong ($|r_p|\geq0.68$). Statistical significance was set at $P\leq0.05$, while P values between >0.05 and <0.10 were considered to indicate a tendency.

Results

The effects of noise levels in the stunning box on oxidative stress biomarkers in slaughter pigs are presented in Table 1. Noise levels in the stunning box significantly affected plasma TOS concentrations (P=0.050) and the oxidative stress index (P=0.050).

Table 1. The effect of noise levels in the stunning box on oxidative stress biomarkers in slaughter pigs (n=60)

Noise level	**Lower (≤105 db)**	**Higher (>105 db)**	***P*-value**	**Significance**
Number of pigs	12	48		
Glucose (mmol/L)	7.39 ± 1.66	7.50 ± 2.28	0.854	ns
AOPP (µM/L)	90.51 ± 16.79	93.12 ±11.21	0.617	ns
Ceruloplasmin (mg/dL)	32.93 ± 8.15	29.22 ± 12.26	0.219	ns
GSH (µmol/L)	0.70 ± 1.24	0.64 ± 1.37	0.868	ns
TAC (mmol/L)	0.69 ± 0.31	0.63 ± 0.39	0.614	ns
TOS (µmol/L)	93.31 ± 18.24	104.20 ± 64.95	0.049	*
Oxidative stress index	0.19 ± 0.07	0.32 ± 0.04	0.046	*

* Statistical significance P<0.05; t: tendency (0.05 <P< 0.10); ns: not statistically significant (P>0.10)

The effects of noise levels in the stunning box on meat quality traits of slaughter pigs are shown in Table 2. Noise levels in the stunning box significantly affected meat pH (P=0.018) and temperature (P=0.023) measured 45 minutes post-slaughter (in the *musculus longissimus lumborum*), cooking loss (P=0.002), sensory color score (P=0.050), as well as $L*$ (P=0.029), $a*$ (P<0.001) and $b*$ (P<0.001) values. In addition, slaughter pigs exposed to lower noise levels (≤105 dB) produced a higher percentage of RFN meat (P=0.048) and a lower frequency of PFN meat (P=0.050).

Pearson correlations between noise level in the stuning box and blood indicators in slaughter pigs are shown in Table 3. No associations (P>0.10) were found between noise level in the stunning box and blood glucose concentration, AOPP, ceruloplasmin, GSH, TAC, TOS and the oxidative stress index.

Table 2. The effects of noise levels in the stunninhg box on pork quality characteristics (n = 60)

Noise level	**Lower (≤105 db)**	**Higher (>105 db)**	***P*-value**	**Significance**
Number of pigs	12	48		
Physicochemical traits				
Musculus longissimus lumborum				
pH_{45min}	6.53 ± 0.18	6.37 ± 0.24	0.018	*
T_{45min} (°C)	35.12 ± 1.70	33.77 ± 1.36	0.023	*
pH_{24h}	5.60 ± 0.28	5.66 ± 0.23	0.464	ns
T_{24h} (°C)	6.61 ±0.55	5.17 ± 1.11	<0.001	*
Musculus semimembranosus				
pH_{45min}	6.52 ± 0.23	6.57 ± 0.21	0.548	ns
T_{45min} (°C)	34.63 ± 2.81	33.39 ± 2.32	0.176	ns
pH_{24h}	5.66 ± 0.13	5.58 ± 0.20	0.077	t
T_{24h} (°C)	6.74 ± 0.50	5.57 ± 1.10	<0.001	*
Water-holding capacity traits (%)				
Drip loss	1.54 ± 0.61	1.97 ± 1.06	0.077	t
Thawing loss	2.81 ± 1.15	2.89 ± 3.31	0.894	ns
Cooking loss	16.23 ± 3.58	20.76 ± 4.98	0.002	*
Pork color traits				
*L** value	46.76 ± 2.68	48.84 ± 2.85	0.029	*
*a** value	7.04 ± 1.70	4.16 ± 1.56	<0.001	*
*b** value	5.93 ± 0.99	7.69 ± 1.12	<0.001	*
Sensory color score	2.65 ± 0.63	2.24 ± 0.60	0.050	*
Pork quality classes				
Moderate PSE meat	0.00	8.16	>0.999	ns
RFN meat	81.82	46.94	0.048	*
PFN meat	0.00	30.61	0.050	*
Moderate DFD meat	18.18	14.29	0.664	ns

* Statistical significance $P<0.05$; t: tendency ($0.05 < P < 0.10$); ns: not statistically significant ($P>0.10$)

Table 3. Association between noise level in the stuning box and blood indicators and meat quality traits in slaughter pigs (n=60)

	Noise level (db)		
	r_p	*P*-value	Strength
Oxidative stress indicators			
Glucose (mmol/L)	0.013	0.921	-
AOPP (µM/L)	0.095	0.470	-
Ceruloplasmin (mg/dL)	-0.185	0.156	-
GSH (µmol/L)	-0.005	0.972	-
TAC (mmol/L)	-0.044	0.739	-
TOS (µmol/L)	0.153	0.188	-
Oxidative stress index	0.134	0.306	-

* Statistical significance $P<0.10$

Pearson correlations between noise level in the stuning box and meat quality traits in slaughter pigs are shown in Table 4. Weak positive correlations were found between noise level and drip loss (*P*=0.050), cooking loss (*P*=0.010), and the *L** (*P*=0.039) values. Noise level in the stunning box showed weak to moderate negative correlation with the meat pH (*P*=0.025) (measured 45 minutes post-slaughter in the *musculus longissimus lumborum*), and *a** value (*P*=0.001). Additionally, noise level in the stunning box moderately positively corelated with meat temperature measured 45 minutes post-slaughter in the *musculus longissimus lumborum* (*P*=0.004) and *b** value (*P*=0.001). Moderate negative correlations were found between noise level and meat temperature measured 24 hours post-mortem in the *musculus longissimus lumborum* (*P*=0.001) and *musculus semimembranosus* (*P*=0.003). Furthermore, no associations (*P*>0.10) were observed between noise level in the stunning box and meat pH measured 24 hours post-mortem in the *musculus longissimus lumborum* and *musculus semimembranosus*, meat pH and temperature measured 45 minutes post-mortem in the *musculus semimembranosus*, thawing loss, and sensory colour score.

Table 4. Association between noise level in the stuning box and meat quality traits in slaughter pigs (n=60)

	Noise level (db)		
	r_p	*P*-value	Strength
Phyisicochemical traits			
Musculus longissimus lumborum			
pH_{45min}	-0.289*	0.025	weak
T_{45min} (°C)	0.371*	0.004	moderate
pH_{24h}	0.078	0.553	-
T_{24h} (°C)	-0.447*	0.001	moderate
Musculus semimembranosus			
pH_{45min}	0.057	0.666	-
T_{45min} (°C)	0.226	0.082	-
pH_{24h}	0.219	0.092	-
T_{24h} (°C)	-0.375	0.003	moderate
Water-holding capacity traits (%)			
Drip loss	0.246*	0.050	weak
Thawing loss	-0.001	0.989	-
Cooking loss	0.329*	0.010	weak
Meat color indicators			
*L** value	0.267*	0.039	weak
*a** value	-0.555	0.001	moderate
*b** value	0.551	0.001	moderate
Sensory color score	-0.236	0.070	-

* Statistical significance *P*<0.10

Discussion

In this study, pigs exposed to higher noise levels (>105 dB) in the stunning box had higher blood plasma TOS concentrations and a higher oxidative stress index (Table 1). These findings indicate that intense acoustic stimuli act as a significant stressor just prior to slaughter that compromises animal welfare by impairing antioxidant defence and induction of oxidative stress (Abuelo et al., 2015). Acute stress immediately before slaughter activates neuroendocrine stress pathways, involving stimulation of the sympathetic–adrenal–medullary and hypothalamic–pituitary–adrenal axes and leading to pronounced physiological, biochemical, and oxidative alterations (Kanitz et al., 2005; Čobanović et al., 2024, 2025), which explaining the elevated oxidative stress indicators recorded in pigs exposed to higher noise levels. Previous studies (Geverink et al., 1998; Rabaste et al., 2007) have also shown that a noisy environment shortly before slaughter (82–108 dB), generated by machinery, pressure hoses and pig and human vocalisations, acts as a significant stressor and induces fear-related behaviours such as huddling and escape attempts in pigs.

Additionally, meat obtained from pigs exposed to the same noise levels (>105 dB) in the stunning box exhibited a higher pH and temperature in the *musculus longissimus lumborum* measured 45 minutes post-mortem, lower water-holding capacity (greater cooking loss), and paler colour (higher *L** and *b** values, lower *a** value and sensory colour score), which consequently resulted in a higher incidence of PFN meat (Table 2). Unlike classical PSE development, which is driven by rapid pH decline at high muscle temperatures following severe acute stress, the predominance of PFN meat observed in this study indicates a stress pattern that slows post-mortem metabolism, caused partial denaturation of sarcoplasmic and myofibrillar proteins, while still negatively affecting meat appearance and processing traits (Kovačević et al., 2025). Earlier studies (Talling et al., 1996; Vermeulen et al., 2015, 2016) reported that exposure of pigs to intense noise (>85 dB) activates stress defence mechanisms, increasing heart rate and muscle temperature, which accelerates early post-mortem glycolysis, resulting in lower early meat pH and a higher incidence of PSE meat (Talling et al., 1996; Vermeulen et al., 2015, 2016). The differences observed suggest that pigs in the present study, despite exposure to higher noise levels, experienced mild stress that slowed post-mortem muscle metabolism and induced partial denaturation of sarcoplasmic and myofibrillar proteins, resulting in a lower incidence of PSE and a predominance of PFN and RFN meat, a pattern distinct from classical PSE development driven by rapid pH decline under severe acute stress (Čobanović et al., 2021; Kovačević et al., 2025). The lack of association between noise level and most systemic oxidative stress biomarkers (Table 3) suggests that the stress induced in the stunning box was localized and short-term, primarily influencing muscle metabolism rather than causing a generalized oxidative imbalance. Nevertheless, the detected weak to moderate correlations between noise intensity and pork pH, temperature, drip loss, and colour parameters (Table 4) confirm that even moderate welfare impairments immediately before slaughter can translate into measurable deterioration of meat quality traits. These results support the hypothesis that mild to moderate pre-slaughter stress, such as excessive noise, may reduce the incidence of PSE meat while increasing PFN meat frequency, which still represent economically undesirable outcomes due to poor visual quality and reduced processing suitability (Čobanović et al., 2021). Based on the results of this study, it can be argued that minimising noise exposure in stunning facilities is essential not only to safeguard animal welfare but also to optimize post-mortem muscle biochemistry and ensure consistent pork quality, reinforcing the strong welfare–quality interconnection in modern meat production systems.

Conclusion

The results of this study showed that measuring noise levels in the stunning box has potential to serve as a non-invasive tool for assessing well-being conditions and variations in pork quality. However, although a degree of association was established between noise level and certain oxidative stress biomarkers and meat quality traits the correlation coefficients were relatively low. Therefore, to more robustly validate the use of noise level as an indicator for monitoring welfare conditions and variation in meat quality, additional studies conducted under diverse pre-slaughter conditions and involving larger sample sizes are required.

Pre-mortem određivanje nivoa buke: neinvazivna metoda za ocenu dobrobiti i kvaliteta mesa svinja

Mina Ilić, Branko Suvajdžić, Đorđe Pajičić, Milena Radaković, Ružica Trailović, Nikola Čobanović

Rezime

Cilj ovog istraživanja bio je da se ispita uticaj nivoa buke u boksu za omamljivanje na pokazatelje oksidativnog stresa i kvaliteta mesa svinja. Ispitivanja su obavljena na 60 svinja, starosti oko šest meseci, prosečne telesne mase oko 110 kg. Kod svake jedinke, određivani su pokazatelji oksidativnog stresa (koncentracija glukoze, AOPP, ceruloplazmina, GSH, UAK, UOS, kao i indeks oksidativnog stresa) i kvalitet mesa (pH vrednost i temperatura, boja mesa, sposobnost vezivanja vode i klase kvaliteta mesa). Nivo buke iznad 105 dB značajno je uticao na koncentraciju UOS u krvnoj plazmi, indeks oksidativnog stresa, kao i na pH vrednost i temperaturu mesa (*musculus longissimus lumborum*) 45 minuta post-mortem, kalo kuvanja, *L**, *a** i *b** vrednosti instrumenatlno određene boje mesa, senzornu ocenu za boju mesa i na učestalost crvenog, čvrstog i nevodnjikavog i bledog, čvrstog i nevodnjikavog mesa. Na osnovu rezultata ovog istraživanja može se zaključiti da se određivanje nivoa buke može potencijalno koristiti za ocenu dobrobiti i kvaliteta mesa svinja.

Ključne reči: dobrobit životinja, nivo buke, kvalitet mesa, oksidativni stres.

Acknowledgement
The study was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia (Contract number 451-03-34/2026-03/200143).

Conflict interest
No potential conflict of interest was reported by the authors.

References

Abuelo A., Hernández J., Benedito J.L., Castillo C. 2015. The importance of the oxidative status of dairy cattle in the periparturient period: revisiting antioxidant supplementation. *Journal of Animal Physiology and Animal Nutrition,* 99, 1003-1016.

Čobanović N., Stanković S.D., Dimitrijević M., Suvajdžić B., Grković N., Vasilev D., Karabasil N. 2020. Identifying physiological stress biomarkers for prediction of pork quality variation. *Animals*, 10(4), 614.

Čobanović N., Novaković S., Tomašević I., Karabasil N. 2021. Combined effects of weather conditions, transportation time and loading density on carcass damages and meat quality of market-weight pigs. *Archives Animal Breeding,* 64(2), 425-435.

Čobanović N., Čalović S., Suvajdžić B., Grković N., Stanković S.D., Radaković M., Spariosu K., Karabasil N. 2024. Consequences of transport conditions on the welfare of slaughter pigs with different health status and RYR-1 genotype. *Animals*, 14(2), 191.

Čobanović N., Grković N., Vićić I., Radaković M., Spariosu K., Stanković S.D., Karabasil N. 2025. Relationship between skin lesion severity and physiometabolic blood profile, microbial counts and carcass and meat quality characteristics in slaughtered pigs. *Meat Science,* 229:109923.

Correa J.A., Methot S., Faucitano L. 2007. A modified Meat Juice Container (EZ-Driploss) Procedure for a more Reliable Assessment of Drip Loss and Related Quality Changes in Pork Meat. *Journal of Muscle Foods*, 18, 67-77.

Erel O. 2004. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clinical Biochemistry,* 37(4), 277-285.

Erel O. 2005. A new automated colorimetric method for measuring total oxidant status. *Clinical Biochemistry,* 38(12), 1103-1111.

Geverink N.A., Bühnemann A., van de Burgwal J.A., Lambooij E., Blokhuis H.J., Wiegant V.M. 1998. Responses of slaughter pigs to transport and lairage sounds. *Physiology and Behavior,* 63(4), 667-673.

Grandin T. 1996. Factors that impede animal movement at slaughter plants. *Journal of the American Veterinary Medical Association,* 209(4), 757-759.

Honikel K.O. 1998. Reference methods for the assessment of physical characteristics of meat. *Meat Science,* 49(4), 447-457.

Hunt M.C., Acton J.C., Benedict R.C., Calkins C.R., Comforth D.P., Jeremiah L.E., Olson D.G., Salm C.P., Savell J.W., Shivas S.D. 1991. Guidelines for meat colour evaluation. American Meat Science Association and National Live Stock and Meat Board, Chicago, Illinois, USA.

Hussein H.A., Bäumer J., Staufenbiel R. 2019. Validation of an automated assay for measurement of bovine plasma ceruloplasmin. *Acta Veterinaria Scandinavica,* 61, 34.

Jollow D.J., Mitchell J.R., Zampaglione N., Gillette J.R. 1974. Bromobenzene-Induced Liver Necrosis. Protective Role of Glutathione and Evidence for 3,4-Bromobenzene Oxide as the Hepatotoxic Metabolite. *Pharmacology,* 11(3), 151–169.

Kanitz E., Otten W., Tuchscherer M. 2005. Central and peripheral effects of repeated noise stress on hypothalamic–pituitary–adrenocortical axis in pigs. *Livestock Production Science,* 94(3), 213-224.

Klauke T.N., Piñeiro M., Schulze-Geisthövel S., Plattes S., Selhorst T., Petersen B. 2013. Coherence of animal health, welfare and carcass quality in pork production chains. *Meat Science,* 95(3), 704-711.

Kovačević S., Grković N., Suvajdžić B., Sindjić M., Dimitrijević V., Becskei Z., Čobanović N. 2025. Classification of broiler breast meat: Defining red, soft and exudative meat as a new quality class. *Poultry,* 4(4), 57.

National Pork Producers Council 2000. Pork Composition and Quality Assessment Procedures. NPPC, Des Moines, IA.

Rabaste C., Faucitano L., Saucier, L., Mormède P., Correa J.A., Giguère A., Bergeron R. 2007. The effects of handling and group size on welfare of pigs in lairage and their influence on stomach weight, carcass microbial contamination and meat quality. *Canadian Journal of Animal Science,* 87(1), 3-12.

Sanchez P.D.C., Arogancia H.B.T., Boyles K.M., Pontillo A.J.B., Ali M.M. 2022. Emerging non-destructive techniques for the quality and safety evaluation of pork and beef: Recent advances, challenges, and future perspectives. *Applied Food Research,* 2, 2, 100147.

Svoboda M., Nemeckova M., Medkova D., Sardi L., Hodkovicova N. 2024. Non-invasive methods for analysing pig welfare biomarkers. *Veterinarni Medicina,* 69(5), 137.

Talling J.C., Waran N.K., Wathes C.M., Lines J.A. 1996. Behavioural and physiological responses of pigs to sound. *Applied Animal Behaviour Science*, 48(3–4), 187-201.

Vermeulen L., Van de Perre V., Permentier L., De Bie S., Verbeke G., Geers R. 2015. Sound levels above 85 dB pre-slaughter influence pork. *Meat Science*, 100(2), 269-274.

Vermeulen L., Van de Perre V., Permentier L., De Bie S., Verbeke G., Geers R. 2016. Pre-slaughter sound levels and pre-slaughter handling from loading at the farm till slaughter influence pork quality. *Meat Science*, 116(6), 86-90.

Witko-Sarsat V., Friedlander M., Capeillère-Blandin C., Nguyen-Khoa T., Nguyen A. T., Zingraff J., Jungers P., Descamps-Latscha B. 1996. Advanced oxidation protein products as a novel marker of oxidative stress in uremia. *Kidney International*, 49(5), 1304-1313.

Received 3 December 2025; accepted for publication 10 March 2026

Biotechnology in Animal Husbandry 42(1), 99-118, 2026
Publisher: Institute for Animal Husbandry, Belgrade-Zemun

ISSN 1450-9156
UDC 633.32'631.563
https://doi.org/10.2298/BAH2601099C

LACTIC ACID BACTERIA AND ENZYME INOCULATION OF ITALIAN RYEGRASS-RED CLOVER SILAGE: ENHANCING MICROBIAL PROFILE, FERMENTATION QUALITY, AND DIGESTIBILITY

Emel Cam[1], **Zehra Selcuk**[2]

[1] Ondokuz Mayıs University, Graduate School, Department of Animal Nutrition and Nutritional Diseases, 55200, Samsun, Türkiye
[2] Ondokuz Mayıs University, Faculty of Veterinary Medicine, Department of Animal Nutrition and Nutritional Diseases, 55200, Samsun, Türkiye
Corresponding author: Zehra Selçuk, zselcuk@omu.edu.tr
Original scientific paper

Abstract: This study evaluated the effects of inoculating Italian ryegrass-red clover with a commercial additive containing lactic acid bacteria and enzymes (LABE) on the microbial profile, fermentation quality, nutritive value, and digestibility of silages. The experimental treatments were: (1) control (without LABE); (2) LABE1 (inoculated at 1.5×10^5 cfu/g of ensiled mass); and (3) LABE2 (inoculated at 3.0×10^5 cfu/g of ensiled mass). After a 60-day incubation period, nutrient composition, fermentation characteristics, microbiology, and digestibility were determined. The pH values of the first harvest Italian ryegrass-red clover silages were 4.59, 4.15, and 4.14 for the control, LABE 1, and LABE 2 groups, respectively (P<0.05), while the corresponding values for the second harvest silages were 4.90, 4.09, and 4.14 (P<0.05). In the control silages, the lactic acid contents were 7.60 and 3.68%, for the first and second harvests, respectively. Following LABE inoculation, these values increased to 8.02 and 10.01% for the first harvest LABE 1 and LABE 2 groups, and to 6.15 and 6.38% for the second harvest treatments, respectively (P<0.05). Ammonia nitrogen concentrations did not differ in the first harvest, however, in the second harvest, the LABE2 group had higher levels (101.54 g/L) compared to control (69.66 g/L) and LABE1 (81.25 g/L) groups (P<0.05). Microbiological analysis showed a decrease (P<0.05) in total aerobic mesophilic bacteria and enterococci populations in both harvests, while lactic acid bacteria counts were unaffected. Yeast-mold, and enterobacteria counts were below detection limits (<2.3 $\log_{10}$ cfu/g) in all groups. Furthermore, the *in vitro* neutral detergent fiber digestibility values of the first and second harvest control silages were 34.97 and 32.68%, respectively. LABE inoculation increased these values to 41.13 and 35.04% for the first harvest LABE 1 and LABE 2 groups, and to 42.45 and 39.26% for the second harvest treatments, respectively (P<0.05).

The population of lactic acid bacteria present in the natural microflora of fresh forage is often unpredictable. By overcoming this limitation, LABE improved the overall silage profile, as the enzymes accelerated fiber degradation, providing essential substrates for lactic acid bacteria. Ultimately, the LABE 2 treatment (inoculated at 3.0×10 cfu/g) produced the most optimal results. Consequently, these improvements in fiber digestibility are expected to positively affect overall animal performance, although further *in vivo* research is required to confirm these effects.

Key words: digestibility, fermentation, inoculant, Italian ryegrass-red clover silage, microbial profile

Introduction

The primary objective of silage production is to preserve green forage with high nutritional value while minimizing nutrient loss. In this process, in addition to the chemical composition of the ensiled mass, the microbial population and environmental conditions that stimulate or inhibit its growth significantly influence silage quality (Ávila and Carvalho, 2019). To ensure and enhance fermentation, the use of silage additives is generally recommended. Microbial inoculants and enzymes are increasingly used, as they are recognized as safe, natural products that do not pose environmental risks (Irawan et al., 2021; Liu et al., 2021). The addition of lactic acid bacteria (LAB) inoculants protect silage from spoilage by enhancing lactic acid (LA) fermentation, lowering pH, and thereby inhibiting the growth of undesirable microorganisms, ultimately improving its nutritional value (Wang et al., 2019). While inoculants primarily consist of LAB, whose main function is to rapidly reduce pH, decrease proteolysis, and increase the aerobic stability of the silage, enzymes are added to this mixture to degrade structural fibers. This enzyme addition increases carbohydrate availability, working synergistically with the bacterial combinations to enhance overall fermentation quality (Dong et al., 2025).

Italian ryegrass is a globally cultivated forage crop, widely grown in temperate, subtropical, and tropical plateau regions, and its silage is extensively used for feeding ruminants during the winter months (Yin et al., 2022). Green forage yields for the first, second, and third harvests are approximately 8.65, 8.48, and 9.21 t/ha, respectively, resulting in a total green herbage production of 26.34 t/ha (Şahin, 2019).

Red clover is a versatile temperate forage crop cultivated extensively for hay, grazing, and silage production (McKenna et al., 2018). It is highly valued for its ease of establishment, rapid growth, high forage quality, and excellent soil improvement characteristics across a wide range of environmental conditions. Its maximum dry matter yield during the first harvest year in Europe can range from 9 to 18 t/ha without nitrogen fertilization, although these yields tend to decrease sharply after the second harvest year. A major nutritional advantage of red clover is that its digestibility declines more slowly with advancing maturity compared to grasses. Therefore, mixing red clover with grass species extends the optimal harvest window, yielding a highly digestible forage ideal for high-yielding dairy cows. Due to these characteristics, red clover is most commonly preserved as silage for the winter feeding of ruminants (Frankow-Lindberg, 2017).

Leguminous forages such as red clover are rich in protein but often lack sufficient water-soluble carbohydrates (WSC) for optimal LA production during ensiling. Furthermore, their high buffering capacity can delay pH decline, thereby increasing the risk of undesirable fermentation. Recent studies have confirmed that the fermentation dynamics and microbial community structure of red clover silage are highly sensitive to substrate composition and initial epiphytic microbiota (Dong et al., 2022). To overcome these limitations, mixing legumes with gramineous forages such as Italian ryegrass has been widely adopted as an effective strategy to improve fermentation quality and nutrient balance (Dündar and Mut, 2023). However, recent research on Italian ryegrass silage has shown that fermentation characteristics are strongly influenced by factors such as maturity stage and microbial composition, indicating that optimizing silage mixtures remains complex and system-dependent (Yin et al., 2023). In parallel, the use of silage additives has advanced considerably in recent years, with growing interest in combined microbial and enzymatic treatments. Recent studies have demonstrated that the synergistic use of LAB and fibrolytic enzymes can enhance fermentation quality, promote fiber degradation, and modify microbial communities more effectively than single additives (Liu et al., 2024; Fang et al., 2025). However, these studies have largely focused on low-quality crop residues or single-species silages, and their applicability to legume-grass mixtures remains unclear. More importantly, there is still limited information regarding the use of combined LAB and enzyme (E) inoculants (LABE) in Italian ryegrass-red clover silages, particularly concerning their integrated effects on fermentation dynamics, fiber degradation, and digestibility. Therefore, a clear research gap exists regarding the effectiveness of LABE inoculants in optimizing both fermentation quality and nutrient utilization in legume- gramineous silage systems. This study aims to address this gap by evaluating the effects of a LABE inoculant on the fermentation characteristics, fiber degradation, and digestibility of Italian ryegrass-red clover silage. We hypothesized that LABE application would enhance fermentation efficiency through accelerated acidification and improve digestibility by promoting plant cell wall degradation.

Materials and Methods

Silage material and experimental design

The experiment was conducted in a field in Amasya Province in the Middle Black Sea Region of Turkey (41˚ 2’ 5.48″ E and 36˚ 14’ 41.97″ N). A mixture of Italian ryegrass (*Lolium multiflorum* cv. Jivet) and red clover (*Trifolium pratense* cv. Suez), consisting of 80% and 20% seeds, respectively, was cultivated and sown at a rate of 4 kg/ha. The forage was harvested at the milk stage of Italian ryegrass, which served as the reference maturity point for the mixture (Mut et al., 2020). After harvesting, the forage from two separate harvests was wilted and chopped into pieces approximately 1.5 cm long.

The experimental treatments and the detailed composition of the commercial inoculant (Sil All 4×4, Lallemand Animal Nutrition, Blagnac, France; Lot No: V3136; Prod: 02/02/2020) were presented in Table 1.

Table 1. Experimental treatments and inoculant characteristics

	Control	LABE1	LABE2
Inoculant rate (cfu/g)	None	1.5×10^5 cfu/g of ensiled mass	3.0×10^5 cfu/g of ensiled mass
Inoculant	LAB: *Lactobacillus plantarum* CNCM l-3235, *Pediococcus pentosaceus* NCIMB 12455, *Pediococcus acidilactici* CNCM l-3237, *Propionibacterium acidipropionici* CNCM MA26/4 E: alpha amylase from *Bacillus amyloliquefaciens*, cellulase from *Trichoderma reesei*, xylanase from *Trichoderma longibrachiatum*, beta-glucanase from *Aspergillus niger*		

To ensure uniform distribution, the inoculant was first mixed into a minimal volume of distilled water, then applied to the chopped forage using a fine spray technique followed by vigorous mixing. For each treatment, the chopped forage mixtures were packed into laboratory-scale glass jar silos with a capacity of 1 L. Proper compaction to achieve optimal anaerobic conditions is essential for successful fermentation and minimizing aerobic spoilage. Following DLG (2012) guidelines, a target packing density of 200-240 kg dry matter (DM)/m³ is recommended. In this study, a density of 240 ± 5 kg DM/m³ was achieved, ensuring high-quality preservation and a stable fermentation environment. To prevent oxygen ingress and maintain anaerobic conditions, the glass jar silos were tightly filled with chopped forage and sealed using screw caps combined with plastic tape. A total of 15 silos were used for each harvest period, providing five replicates per experimental group. The silos were then stored in a temperature-controlled environment at 21±2°C for a 60-day fermentation period.

Chemical analysis of silages

Silage materials and also silage samples collected from the silos were dried at 60°C for 48 hours in a circulating air oven and ground to a 1 mm particle size. The DM, organic matter (OM), and crude protein (CP) contents were analysed using standard methods of AOAC (2006). All results for chemical composition and nutrient content were expressed on a DM basis. Fiber fractions, including ash-free acid detergent fiber (ADF_{OM}), ash-free neutral detergent fiber (NDF_{OM}), and ash-free acid detergent lignin (ADL_{OM}), were determined using an Ankom 200/220 Fiber Analyzer according to Van Soest et al. (1991). Total digestible nutrients (TDN), non-fiber carbohydrates (NFC), and total carbohydrates (TC) were calculated using the following equations:

TDN (%) = 105.2 – 0.68 × %NDF (Chandler, 1990).

NFC (%) = 100 – (%NDF + %CP + %Ether Extract (EE) + %Ash) (Weiss et al., 1992).

TC (%) = 100 – (%CP + %EE + %Ash) (Sniffen et al., 1992).

Determination of fermentation characteristics of silages

After the 60-day ensiling period, in order to determine the pH value of the experimental silages, a 25 g silage sample from each silo was homogenized in 100 mL of distilled water for 10 minutes (Polan et al., 1998). The pH of the silage extract was measured using a pH meter (Thermo Orion 710 A+, Thermo Electron Corporation). The LA concentrations in the silages were determined spectrophotometrically using the Barnett method (Barnett, 1951; Tekin and Kara, 2020). A standard lithium lactate curve (0.312–160 µg/ml; $R^2 = 0.95$) was used to calculate lactate equivalents. To express the results as a percentage of silage DM, the following equation was applied:

$$\text{LA, (\%) in DM} = \frac{\text{absorbance value} \times 10^{-2} \times (100 - \text{DM})}{\text{DM}}$$

For the analysis of volatile fatty acids, 1.5 mL of silage extract was acidified with 0.3 mL of 25% (w/v) meta-phosphoric acid. After centrifugation at 15,000 rpm for 15 minutes, the supernatant was transferred to vials for gas chromatography. Acetic (AA), butyric (BA), and propionic (PA) acid concentrations were measured using a Thermo Trace 1300 GC system coupled with an AI-1310 autosampler (Ersahince and Kara, 2017). Final values were quantified using Xcalibur software, with peaks identified by retention times and calculated as a percentage of the silage DM.

Determination of ammonia concentration in silages

Ammonia (NH_3-N) levels in the silage extract were quantified using a commercial enzymatic kit (K-AMIAR, Megazyme, Wicklow, Ireland). The analytical procedure involved mixing 0.10 mL of silage fluid with 2.0 mL of distilled water, then adding 0.3 mL of buffer (containing 0.02% w/v sodium azide and 2-oxoglutarate) and 0.2 mL of NADPH. Absorbance was measured at 340 nm using an UviLine 9100 spectrophotometer (Xylem Analytics Germany Sales GmbH & Co. KG, Mainz, Germany). After the initial measurement, a glutamate dehydrogenase suspension was added to the mixture, and the absorbance was measured again at 340 nm. The final ammonia concentration (g/L) was calculated according to the protocol provided by the manufacturers.

Microbiological analysis

Total aerobic mesophilic bacteria (TAMB), LAB, enterobacteria, enterococcus, yeasts and molds counts in the silages were determined using Plate Count Agar (PCA), de Man, Rogosa and Sharpe Agar (MRS), Violet Red Bile Glucose (VRBG) Agar, Slanetz Bartley Medium (SBM), and Rose Bengal Medium Agar (RO) (chloramphenicol selective supplement agar), respectively. Ten grams of silage were sampled and blended with 90 mL of sterilized distilled water containing peptone in sterile-filtered bags using a stomacher (Lab Blender 400, Sewart-England), then serially diluted in sterilized distilled water from 10^{-1} to 10^{-6}. To determine the number of TAMB, PCA was incubated at 37 ºC for 48 h. The number of LAB was determined by using MRS agar incubated at 30 ºC for 48 h in an anaerobic environment. Both VRBG for enterobacteria and SBM for enterococcus were incubated at 37 ºC for 48 h. RO agar for yeast and mold was incubated at 25 ºC for 5-7 days. Visible colonies were counted from plates at appropriate dilutions, and the number of colony forming units (cfu) was expressed per gram of fresh sample.

Determination of *in vitro* digestibility values

Rumen inoculum was obtained from adult cattle at a local slaughterhouse in Samsun. Following collection, the fluid was filtered through four layers of cheesecloth and transferred into a CO_2-flushed thermos maintained at 39°C. The initial pH was measured at 6.25. For the digestibility analysis, approximately 0.5 ± 0.05 g of silage material and silage samples were weighed into Ankom F57 filter bags, which were subsequently completely closed using a heat sealer. In accordance with the Ankom Daisy incubator's operational protocol, a 48-hour incubation was performed to determine various *in vitro* metrics, specifically *in vitro* true digestibility (IVTD), *in vitro* true dry matter digestibility (IVTDMD), *in vitro* true organic matter digestibility (IVTOMD), and *in vitro* NDF digestibility (IVNDFD) % (Kılın and Selçuk, 2024).

Statistical analysis

Data were analyzed using one-way ANOVA considering treatment as the main effect. The normality of the data distribution was tested using the Shapiro-Wilk and Kolmogorov-Smirnov tests. Levene's test was used to assess the homogeneity of variances. Mean values that were normally and homogeneously distributed were compared by one-way ANOVA followed by Tukey’s test. Non-homogeneous mean values were compared using Tamhane’s test. For non-normal distributions, the non-parametric Kruskal-Wallis test was used to compare means, and the Mann-Whitney test was subsequently applied to identify the sources of significant differences. Statistical analyses were performed using SPSS (Version 21.0, IBM Corp., Armonk, NY, USA). Differences among means were considered statistically significant at $P<0.05$.

Results and Discussion

In this study, the separate evaluation of each harvest provided a more accurate interpretation of inoculant effects, as variations in chemical composition between harvests are known to influence fermentation dynamics and treatment responses. Accordingly, the effects of LABE treatments were interpreted within each harvest to account for these compositional differences.

Nutrient composition and fermentation characteristics of silages

The nutrient and chemical compositions of the first and second harvest Italian ryegrass-red clover silages are presented in Table 2.

Clear differences between the chemical composition of the fresh forage and the resulting silages indicate that ensiling substantially modified nutrient fractions, largely depending on harvest. Although silage DM generally reflected that of the initial material, first-harvest silages exhibited slightly lower DM than the fresh forage, suggesting greater fermentation losses under high-moisture conditions. In contrast, second-harvest silages more closely preserved the initial DM content, likely due to reduced fermentation and effluent losses in drier forages during ensiling.

Table 2. Initial chemical composition and *in vitro* digestibility of first and second harvest Italian ryegrass-red clover green mass prior to ensiling, expressed as % of DM.

	The first harvest	The second harvest
Chemical composition, %		
Fresh DM	28.54	38.89
Ash	15.53	11.4
OM	78.34	80.66
EE	1.94	1.85
CP	16.91	17.63
ADF_{OM}	26.38	27.65
NDF_{OM}	45.79	49.51
ADL_{OM}	2.59	3.67
TDN	74.05	71.53
NFC	19.82	19.61
TC	65.62	69.12
***In vitro* digestibility, %**		
IVTD	74.56	69.27
IVTDMD	72.90	66.62
IVTOMD	68.92	62.79
IVNDFD	40.84	32.47

DM: Dry matter, OM: Organic matter, EE: Ether extract, CP: Crude protein, ADF_{OM}: Acid detergent fiber-ash free, NDF_{OM}: Neutral detergent fiber-ash free, ADL_{OM}: Acid detergent lignin-ash free, TDN: Total digestible nutrients, NFC: Non-fiber carbohydrates, TC: Total carbohydrates, IVTD: *In vitro* true digestibility, IVTDMD: *In vitro* true dry matter digestibility, IVTOMD: *In vitro* organic matter digestibility, IVNDFD: *In vitro* neutral detergent fiber digestibility.

CP concentrations remained largely stable between fresh material and silages, suggesting effective protein preservation during ensiling. In the present study, the most pronounced differences between fresh material and silages were observed in fiber fractions. In the first harvest, silage NDF and ADF values were substantially lower than those of the fresh forage, indicating partial degradation of structural carbohydrates during fermentation. In contrast, second-harvest silages showed fiber values much closer to the original material, which may be attributed to the constraining effect of increased lignification on cell wall degradation. The relative stability of ADL further supports that lignin is largely resistant to fermentation and limits the accessibility of digestible fiber. These compositional differences appear to be directly associated with fiber digestibility. The greater reduction in NDF and ADF in first-harvest silages corresponds with the higher IVNDFD observed in the fresh material (40.84% vs. 32.47%), suggesting that less lignified fiber is more susceptible to both ensiling-induced modification and ruminal degradation. Overall, the findings of the present study indicate that ensiling does not merely preserve the nutritional value of forage but selectively modifies fiber fractions, with the extent of these changes being strongly influenced by initial maturity. From a practical perspective, first-harvest forage appears to offer greater potential for improving fiber utilization through ensiling, whereas second-harvest forage largely retains its inherent structural limitations.

Table 3. Chemical composition and fermentation characteristics of the first and second harvest Italian ryegrass-red clover silages, expressed as % of DM

	Control	LABE1	LABE2	SEM	P value
		The first harvest			
Fresh DM, %	33.20[a]	31.67[b]	31.35[b]	0.52	0.011
Ash, %	14.51	14.73	14.48	0.11	0.107
OM, %	76.02[b]	76.62[a]	76.83[a]	0.08	0.001
EE, %	2.92	2.92	2.95	0.05	0.970
CP, %	17.24	17.80	17.25	0.20	0.086
ADF_{OM}, %	23.05	23.29	23.56	0.36	0.433
NDF_{OM}, %	34.76[b]	35.85[ab]	36.35[a]	0.43	0.024
ADL_{OM}, %	1.81[b]	2.08[a]	2.00[a]	0.06	0.025
TDN, %	81.56[a]	80.81[ab]	80.47[b]	0.17	0.024
NFC, %	30.54[a]	28.68[b]	28.95[b]	0.34	0.044
TC, %	65.31	64.54	65.31	0.16	0.073
pH	4.59[a]	4.15[b]	4.14[b]	0.04	0.002
LA, %	7.60[b]	8.02[b]	10.01[a]	0.45	0.004
AA, %	0.12	0.15	0.21	0.02	0.260
BA, %	0.010	0.009	0.009	0.00	0.214
PA, %	0.05[b]	0.04[a]	0.04[a]	0.00	0.008
NH_3-N, g/L	75.53	78.17	76.77	0.67	0.301
		The second harvest			
Fresh DM, %	35.69[b]	36.42[b]	36.83[a]	0.17	0.014
Ash, %	11.61[b]	11.94[ab]	12.12[a]	0.06	0.001
OM, %	81.81[b]	82.56[a]	82.64[a]	0.12	0.011
EE, %	2.82	2.58	2.74	0.45	0.080
CP, %	18.39	18.64	18.20	0.11	0.316
ADF_{OM}, %	27.09	27.44	27.90	0.14	0.073
NDF_{OM}, %	43.89[b]	46.24[a]	46.63[a]	0.38	0.001
ADL_{OM}, %	5.06	5.15	5.12	0.11	0.959
TDN, %	75.34[a]	73.75[b]	73.49[b]	0.26	0.001
NFC, %	23.26[a]	20.57[b]	20.29[b]	0.42	<0.001
TC, %	67.16	66.82	66.97	0.16	0.735
pH	4.90[a]	4.09[b]	4.14[c]	0.10	0.002
LA, %	3.68[b]	6.15[a]	6.38[a]	0.43	<0.001
AA, %	0.37	0.40	0.44	0.022	0.500
BA, %	0.010[a]	0.009[b]	0.009[b]	0.00	0.007
PA, %	0.05[a]	0.04[b]	0.04[b]	0.00	0.010
NH_3-N, g/L	69.66[b]	81.25[b]	101.54[a]	4.89	0.009

DM: Dry matter, OM: Organic matter, EE: Ether extract, CP: Crude protein, ADF_{OM}: Acid detergent fiber-ash free, NDF_{OM}: Neutral detergent fiber-ash free, ADL_{OM}: Acid detergent lignin-ash free, TDN: Total digestible nutrients, NFC: Non-fiber carbohydrates, TC: Total carbohydrates, LA: Lactic acid, AA: Acetic acid, BA: Butyric acid, PA: Propionic acid, NH_3-N: Ammonia nitrogen

The DM content of the 80% Italian ryegrass (caramba)-20% Alexandria clover mixture silages was reported as 33.40% (Demiroğlu Topçu and Kahya, 2023). Oliveira et al. (2018) stated that the DM value of good quality silage was between 25-35%. The results for DM content in the current study are inline with previous studies. Regarding fiber fractions, LABE supplementation did not affect ADF content, and this result is consistent with previous studies (Coskuntuna and Gül, 2020; Marbun et al., 2020; Li et al., 2022; Şen et al., 2022; Kılın and Selçuk, 2024). In contrast, a significant increase in NDF content was observed alongside a decline in NFC values following LABE application. Silage inoculation with homofermentative LAB promotes LA fermentation and stimulates the degradation of non-structural carbohydrates (Zhao et al., 2019). This inverse relationship stems from active fermentation, where LAB preferentially utilize readily fermentable carbohydrates as substrates and could cause a decrease in easily fermentable carbohydrates (Oliveira et al., 2017; Cai et al., 2020). Consequently, the proportional increase in NDF likely reflects the depletion of soluble components. Rapid acidification is a critical factor for successful silage preservation, as it suppresses undesirable microbial activity and limits proteolysis (Pahlow et al., 2003). In this study, CP contents remained largely stable across treatments, which can be attributed to the presence of polyphenol oxidase (PPO) in red clover. It was reported that the presence of PPO enzyme can limit the natural proteolysis of forages mixed with red clover, thus reducing protein degradation during the ensiling process (Sullivan and Hatfield, 2006; Boller et al., 2010). Muck et al. (2018) stated that the use of additives containing PPO may limit protein losses caused by fermentation of forages. Unchanged in NH3-N concentration in the first harvest silages suggests that PPO can produce quinones that bind to proteins, which in turn reduces protein degradability during ensiling (Frankow-Lindberg, 2017). Thus PPO may have contributed to limiting proteolysis, thereby protecting true protein from excessive degradation. Nevertheless, NH_3-N concentrations were higher in the LABE2 treatment, particularly in second-harvest silages. Interestingly, while elevated NH_3-N levels can sometimes indicate clostridial activity (Doğan Daş et al., 2022), the higher concentrations observed in the LABE2 group must be interpreted differently. The absence of BA (<0.1%) and the negligible counts of Enterobacteriaceae (<2.30 $\log_{10}$ cfu/g) confirm that clostridial fermentation did not occur. Instead, the increased NH_3-N is more plausibly a result of enhanced enzymatic degradation of plant cell structures, which facilitated the release of intracellular nitrogenous compounds. The pH values and silage acid profiles further support this, as LAB are highly effective at producing LA, which has a greater acidifying capacity than other organic acids (Kung Jr et al., 2018;

Peng et al., 2021). Lactic acid concentration increased from 7.60 up to 10.01% of DM in the LABE-treated group of in the first harvest, and also 3.68 up to 6.38% of DM in the LABE-treated group of in the second harvest, indicating a more efficient fermentation compared to the control.The consistently low levels of undesirable fermentation products, such as PA and BA, across all treatments confirm that LABE application maintained fermentation stability, and this finding is consistent with the report of Okoye et al. (2023). Typically, the population of lactic acid bacteria present in the natural microflora of fresh forage is often unpredictable or numerically insufficient to rapidly dominate the fermentation process. Taken together, these findings suggest that overcoming this natural limitation through LABE supplementation, particularly at 3.0×10 cfu/g, improved silage quality primarily by enhancing fermentation efficiency and fiber digestibility rather than by altering overall nutrient concentrations. The observed increases in NDF and NH_3-N should therefore be interpreted not as indicators of reduced silage quality, but as consequences of active fermentation and enzymatic structural relaxation that ultimately enhanced nutrient availability for rumen microorganisms.

Microbiologic properties of silages

The microbiological characteristics of the first and second harvest Italian ryegrass-red clover silages are given in Table 4.

Table 4. Microbiology of Italian ryegrass-red clover silages (log_{10} cfu/g of fesh silage)

	Control	LABE1	LABE2	SEM	P value
		The first harvest			
TAMB	9.29[a]	7.71[b]	7.61[b]	0.24	<0.001
Enterobacteria*	<2.30	<2.30	<2.30		
Enterococcus	7.34[a]	6.81[b]	6.65[b]	0.10	<0.001
LAB	7.14	7.47	7.66	0.24	0.150
Yeast-Mold*	<2.30	<2.30	<2.30		
		The second harvest			
TAMB	8.41[a]	7.28[b]	6.73[b]	0.21	<0.001
Enterobacteria*	<2.30	<2.30	<2.30		
Enterococcus	7.07[a]	6.87[ab]	6.52[b]	0.09	0.030
LAB	7.42	8.02	8.06	0.15	0.180
Yeast-Mold*	<2.30	<2.30	<2.30		

TAMB: Total aerobic mesophilic bacteria, LAB: Lactic acid bacteria

Successful silage fermentation requires epiphytic LAB populations to reach at least 5 log cfu/g (Ennahar et al., 2003; Pang et al., 2012). This threshold was achieved in all experimental groups, indicating that the native microbiota and ensiling conditions were sufficient to support effective LA fermentation. These findings are consistent with previous reports demonstrating adequate LAB development in well-managed silages (Ennahar et al., 2003; Pang et al., 2012; Bureenok et al., 2019). Moreover, the absence of a marked treatment effect on LAB counts aligns with the observations of Bureenok et al. (2019), who reported that LAB populations were not significantly influenced by enzyme and/or LAB inoculation. Indicators of silage hygienic quality further supported the effectiveness of the fermentation process. Yeast counts remained below the recommended upper limit of 6 log cfu/g (Kung et al., 2018), and mold counts stayed under the 4 log cfu/g threshold indicative of good production practices (Alonso et al., 2013). The yeast and mold counts in both LABE-treated and control silages are consistent with previous studies (Alonso et al., 2013; Ávila and Carvalho, 2019). These results suggest that rapid acidification during fermentation effectively suppressed the growth of undesirable microorganisms. Similar outcomes have been reported in a study showing that LAB and LAB+E inoculation reduce pH and increase LA concentration, thereby limiting yeast and mold proliferation (Uğurlu et al., 2022). Furthermore, lower yeast and mold counts are indicative of improved aerobic stability, as these microorganisms are primarily responsible for spoilage upon air exposure. Enterobacteria are generally present in poorly preserved silages, and some of those are capable of breaking down proteins, leading to the release of NH_3-N and biogenic amines (McDonald et al., 1991). Therefore, the presence of Enterobacteria in well-preserved silages is generally not expected because rapid LA production inhibits Enterobacteria growth. The negligible counts of Enterobacteriaceae in the current study is in agreement with previous study (Ávila and Carvalho, 2019).

Digestibility values of silages

There was an increase ($p<0.05$) in IVTD, IVTDMD, IVTOMD and IVNDFD in the LABE-supplemented silages (Table 5).

LABE supplementation significantly increased IVTD, IVTDMD, IVTOMD, and IVNDFD in both harvests, indicating a substantial improvement in nutritive quality. Roughage digestibility is a primary indicator of silage value, as it directly influences nutrient availability and animal performance (Şen et al., 2022; Liu et al., 2019). While several studies have reported no significant effects of LAB or enzyme additives on DM or organic matter digestibility (Li et al., 2022; Kılın and Selçuk, 2024; Bureenok et al., 2019; Hristov and McAllister, 2002; Muck et al., 2007; Özdüven et al., 2017) others have demonstrated positive responses, suggesting that the efficacy of such additives may depend on forage type, harvest stage, and additive composition (Weinberg et al., 1995; Ozduven et al., 2010).

Table 5. *In vitro* digestibilty values of the silages, expressed as %

	Control	LABE1	LABE2	SEM	P value
		The first harvest			
IVTD	78.78^{b}	80.08^{a}	80.30^{a}	0.10	0.010
IVTDMD	76.57^{b}	78.20^{a}	78.43^{a}	0.08	<0.001
IVTOMD	73.28^{b}	75.04^{a}	75.26^{a}	0.08	<0.001
IVNDFD	34.97^{b}	41.13^{a}	42.45^{a}	0.60	<0.001
		The second harvest			
IVTD	70.74^{b}	72.07^{a}	72.52^{a}	0.26	<0.001
IVTDMD	69.04^{b}	70.11^{ab}	71.01^{a}	0.27	<0.001
IVTOMD	65.38^{b}	66.71^{ab}	67.73^{a}	0.33	<0.001
IVNDFD	32.68^{c}	35.04^{b}	39.26^{a}	0.79	<0.001

IVTD: *In vitro* true digestibility, IVTDMD: *In vitro* true dry matter digestibility, IVTOMD: *In vitro* organic matter digestibility, IVNDFD: *In vitro* neutral detergent fiber digestibility.

The digestibility results of the present study show a clear positive response to LABE supplementation. The observed enhancement in 48-h IVNDFD is particularly noteworthy and consistent with previous studies (Kılın and Selçuk, 2024; Guo et al., 2020). This improvement is likely due to the partial hydrolysis of plant cell wall components by exogenous enzymes during ensiling, which weakens the structural integrity of the fiber matrix (Getabalew et al., 2022). Similar improvements in cellulose fractions have been reported with LAB applications (Aksu et al., 2004; Addah et al., 2011). The positive response to LABE addition in the present study could be attributed to the breakdown of plant cell wall structures in the silage, which effectively liberates intracellular contents, thereby providing more substrates for rumen microorganisms. Oba and Allen (1999) stated that a 1-unit improvement in IVNDFD was positively associated with increases of 0.17 kg in dry matter intake (DMI) and 0.25 kg in 4% fat-corrected milk yield. In the present study, the increases observed in IVNDFD for for the first harvest silages, LABE1 and LABE2 treatments may increase DMI by approximately 1.05 and 1.27 kg/day and 4% FCM yield by 1.54 and 1.87 kg/day, respectively. For the second harvest silages, the corresponding increases are estimated at 0.40 and 1.12 kg/day for DMI and 0.59 and 1.65 kg/day for milk yield. Based on these relationships, the improvements in IVNDFD observed in LABE1 and LABE2 treatments could potentially translate into meaningful increases in feed intake and milk production compared to the control. In particular, the higher magnitude of IVNDFD improvement in LABE2 indicates a greater potential to enhance animal performance. These findings highlight the practical importance of improving fiber digestibility, as even moderate increases in IVNDFD can have significant impacts on animal productivity.

Conclusion

Inoculating Italian ryegrass-red clover silage with a combination of LAB and E at levels up to 3.0×10 cfu/g significantly improved fermentation characteristics and the microbial profile by significantly suppressing the growth of TAMB and Enterococcus while maintaining the lactic acid bacteria population, and enhanced *in vitro* digestibility. The observed improvements, especially in fiber degradation, show that LABE effectively optimizes the fermentation process and enhances the overall nutritive value of the silage. These findings indicate that LABE is a promising biological additive for grass-based silages, contributing to superior silage quality and greater fermentation stability. Future research should evaluate the effect of LABE-treated Italian ryegrass-red clover silages on animal performance parameters, such as feed intake, nutrient utilization, and milk or meat production. Additionally, investigating different enzyme formulations, inoculation rates, forage types and the interaction between LABE application and harvest maturity will provide deeper insights into optimizing its efficacy under practical farming conditions.

Inokulacija silaže italijanskog ljulja i crvene deteline mlečnokiselinskom bakterijom i enzimima: poboljšanje mikrobnog profila, kvaliteta fermentacije i svarljivosti

Emel Cam, Zehra Selcuk

Rezime

Ova studija procenjuje uticaj inokulacije silaže italijanskog ljulja i crvene deteline komercijalnim aditivom koji sadrži mlečnokiselinske bakterije i enzime (LABE) na mikrobiološki profil, kvalitet fermentacije, nutritivnu vrednost i svarljivost silaže. Eksperimentalni tretmani su se sastojali od: (1) kontrole (bez LABE); (2) LABE1 (inokuliran sa $1{,}5\times10^5$ cfu/g silirane mase); i (3) LABE2 (inokuliran sa $3{,}0\times10^5$ cfu/g silirane mase). Nakon perioda inkubacije od 60 dana, određeni su sastav hranljivih materija, karakteristike fermentacije, mikrobiologija i svarljivost. LABE inokulacija je povećala sadržaj mlečne kiseline ($P<0{,}05$) i u prvom i u drugom otkosu. Koncentracije amonijačnog azota se nisu razlikovale u prvom otkosu, ali LABE2 grupa je pokazala više ($P<0{,}05$) nivoe amonijačnog azota u drugom otkosu u poređenju sa ostalim grupama. Mikrobiološka analiza je otkrila smanjenje ($P<0{,}05$) ukupnih populacija aerobnih mezofilnih bakterija i enterokoka u obe žetve, dok je broj bakterija mlečne kiseline ostao nepromenjen. Broj kvasca, plesni i enterobakterija bio je ispod granica detekcije (<2,3 log10 cfu/g) u svim grupama.

Dodatno, LABE inokulacija je poboljšala vrednosti svarljivosti in vitro (P<0,05) u oba otkosa. Ovi rezultati ukazuju na to da LABE inokulacija u nivoima do $3{,}0\times10^5$ cfu/g silažne mase može poboljšati nutritivnu vrednost, mikrobiološki profil i svarljivost silaže od italijanskog ljulja i crvene deteline. Međutim, potrebna su dalja istraživanja kako bi se procenili njeni efekti na performanse životinja.

Ključne reči: svarljivost, fermentacija, inokulant, silaža od italijanskog ljulja i crvene deteline, mikrobni profil

Acknowledgement
The results of this research were financed by the Scientific Research Projects Coordination Unit of Ondokuz Mayıs University (PYO.VET.1904.22.009). We would like to thank the Scientific Research Projects Coordination Unit of Ondokuz Mayıs University for their support.

Conflict of interest
The authors declare no conflicts of interest.

References

Addah W., Baah J., Groenewegen P., Okine E.K., McAllister T.A. 2011. Comparison of the fermentation characteristics, aerobic stability and nutritive value of barley and corn silages ensiled with or without a mixed bacterial inoculant. *Canadian Journal of Animal Science*, 91(1), 133-146.

Aksu T., Baytok E., Bolat D. 2004. Effects of a bacterial silage inoculant on corn silage fermentation and nutrient digestibility. *Small Ruminant Research,* 55, 249-252.

Alonso V.A., Pereyra C.M., Keller L.A.M., Dalcero A.M., Rosa C.A.R., Chiacchiera S.M., Cavaglieri L.R. 2013. Fungi and mycotoxins in silage: an overview. *Journal of Applied Microbiology,* 115, 637-643.

AOAC. Official Methods of Analysis. 18th.Ed. Association of Official Analytical Chemists, Gaithersburg: MD; 2006.

Ávila C., Carvalho B.F. 2019. Silage fermentation-updates focusing on the performance of microorganisms. *Journal of Applied Microbiology*, 128, 966-984.

Barnett A.J. 1951. The colorimetric determination of lactic acid in silage. *Biochemical Journal*, 49, 527-529.

Boller B., Schubiger F.X., Kolliker R. 2010. Red Clover. In: Boller B., Posselt U.K., Veronesi F. (Eds), *Fodder Crops and Amenity Grasses* (439-455). New York: Springer.

Bureenok S., Langsoumechai S., Pitiwittayakul N., Yuangklang C., Vasupen K., Saenmahayak B., Schonewille J.T. 2019. Effects of fibrolytic enzymes and lactic acid bacteria on fermentation quality and in vitro digestibility of Napier grass silage. *Italian Journal of Animal Science,* 18, 1438-1444.

Cai Y., Du Z., Yamasaki S., Nguluve D., Tinga B., Macome F., Oya T. 2020. Community of natural lactic acid bacteria and silage fermentation of corn stover and sugarcane tops in Africa. *Asian-Australasian Journal of Animal Science,* 33, 1252.

Chandler P. 1990. Energy prediction of feeds by forage testing explorer. *Feedstuffs*, 62(36), 1-12.

Coskuntuna L., Gül S. 2020. The effects of lactic acid bacteria and inoculants mixture enzyme on fermentation and feed value of Vetch *(Vicia narbonensis* L.) silage. *International Journal of Current Research*, 12(3), 10680-10683.

Demiroğlu Topçu G., Kahya M.E. 2023. İskenderiye üçgülü *(Trifolium alexandrium* L.) ile İtalyan çimi (*Lolium multiflorum* Lam.) karışımlarının bazı silaj özelliklerinin belirlenmesi. *Research Journal of Biology Sciences,* 16(1), 8-15

Deutsche Landwirtschafts-Gesellschaft. 2012. *Praxishandbuch Futter- und Substratkonservierung* (8. Aufl.). DLG-Verlag.

Doğan Daş B., Denek N., Baytur G. 2022. Effect of supplementing corn silage with commercial (Bonsilage-Mais) and pre-fermented lactic acid bacteria inoculant on silage quality. *Erciyes Üniversitesi Veteriner Fakültesi Dergisi,* 19(1), 24-29.

Dong L., Zhang H., Diao Q., Wang Y. 2022. Dynamics of fermentation profile, bacterial communities, and their functional characteristics in red clover silage. *Journal of Agricultural Science,* 160(5), 623-635.

Dong D., Zhang L., Zhao J., Dong Z., Li J., Shao T. 2025. Synergistic effects of exogenous *Lactobacillus plantarum* and fibrolytic enzymes on fermentation quality, fiber degradation, and in vitro digestibility of Napiergrass *(Pennisetum purpureum)* silage. *Agronomy,*15, 340.

Dündar A.A., Mut H. 2023. Determination of silage quality of alfalfa and annual ryegrass mixture. *ISPEC Journal of Agriccultural Science,* 7, 739-746.

Ennahar S., Cai Y., Fujita Y. 2003. Phylogenetic diversity of lactic acid bacteria associated with paddy rice silage as determined by 16S ribosomal DNA analysis. *Applied and Environmental Microbiology,* 69(1), 444-451.

Ersahince A.C., Kara K. 2017. Nutrient composition and in vitro digestion parameters of Jerusalem artichoke (*Helianthus tuberosus* L.) herbage at different maturity stages in horse and ruminant. *Journal of Animal and Feed Sciences,* 26, 213-225.

Fang L., Su M., Wu S., Xu W., Weng B., Feng Y., Zhang W., Ma J. 2025. Use of microbial and enzymatic additives on the nutritional quality, fermentation profile, and in vitro digestibility of mixed silages of amaranth and sweet potato vines. *Agriculture*, 15, 2276.

Frankow-Lindberg B. 2017. Red clover in cropping systems. In: Murphy-Bokern D., Stoddard F.L., Watson C.A. (Eds), *Legumes in cropping systems*

(157-167). CAB International.
Getabalew M., Mindaye A., Alemneh T. 2022. Silage and enzyme additives as animal feed animals response. *Archives of Animal Husbandry and Dairy Sciences*, 2(4), 1-6.
Guo G., Shen C., Liu Q., Zhang S.L., Shao T., Wang C., Wang Y.X., Xu Q.F., Huo W.J. 2020. The effect of lactic acid bacteria inoculums on *in vitro* rumen fermentation, methane production, ruminal cellulolytic bacteria populations and cellulase activities of corn stover silage. *Journal of Integrative Agriculture*, 19(3), 838-847.
Hristov A.N., McAllister T.A. 2002. Effect of inoculants on whole-crop barley silage fermentation and dry matter disappearance *in situ. Journal of Animal Science*, 80, 510-516.
Irawan A., Sofyan A., Ridwan R., Hassim H.A., Respati A.N., Wardani W.W., Sadarman Astuti W.D., Jayanegara A. 2021. Effects of different lactic acid bacteria groups and fibrolytic enzymes as additives on silage quality: A meta-analysis. *Bioresource Technology Reports*, 14, 100654.
Kılın Z., Selçuk Z. 2024. Determination of the effect of a lactic acid bacteria+enzyme mixture on the silage quality and digestibility of vetch-oat mixture silage. *Biotechnology in Animal Husbandry*, 40(1), 41-50.
Kung Jr L., Shaver R., Grant R., Schmidt R. 2018. Silage review: Interpretation of chemical, microbial, and organoleptic components of silages. *Journal of Dairy Science*, 101(5), 4020-4033.
Li Y., Du S., Sun L., Cheng Q., Hao J., Lu Q., Ge G., Wang Z., Jia Y. 2022. Effects of lactic acid bacteria and molasses additives on dynamic fermentation quality and microbial community of native grass silage. *Frontiers in Microbiology*, 13, 1-13.
Liu B., Huan H., Gu H., Xu N., Shen Q., Ding C. 2019. Dynamics of a microbial community during ensiling and upon aerobic exposure in lactic acid bacteria inoculation-treated and untreated barley silages. *Bioresource Technology*, 273, 212-219.
Liu C., Zhao G.Q., Wei S.N., Kim H.J., Li Y.F., Kim J.G. 2021. Changes in fermentation pattern and quality of Italian ryegrass (*Lolium multiflorum* Lam.) silage by wilting and inoculant treatments. *Animal Bioscience*, 34(1), 48-55.
Liu E.Y., Wang S., Wang S., Khan N.A., Zhou X., Tang S., Zhou C., Tan Z., Liu Y. 2024. Bacterial inoculants and enzymes based silage cocktails boost the ensiling quality of biomasses from reed, corn and rice straw. *Chemical Biological Technologies Agriculture*, 11(29), 1-19.
Marbun T.D., Lee K., Song J., Kwon C.H., Yoon D., Lee S.M., Kang J., Lee C., Cho S., Kim E. 2020. Effect of lactic acid bacteria on the nutritive value and *in vitro* ruminal digestibility of maize and rice straw silage. *Applied Science*, 10(21), 7801.

McDonald P., Henderson A.R., Heron S.J.E. 1991. *The biochemistry of silage*. Marlow, Bucks, UK: Chalcombe Publications.
McKenna P., Cammon N., Conway J., Dooley J. 2018. The use of red clover *(Trifolium pratense*) in soil fertility-building: A review. *Field Crops Research*, 221, 38-49.
Muck R.E., Filya I., Contreras-Govea F.E. 2007. Inoculant effects on alfalfa silage: in vitro gas and volatile fatty acid production. *Journal of Dairy Science,* 90, 5115-5125.
Muck R.E., Nadeau E.M.G., McAllister T.A., Conteras-Govea F.E., Santos M.C., Kung L. 2018. Silage review: Recent advances and future uses of silage additives. *Journal of Dairy Science,* 101, 3980-4000.
Mut H., Gülümser E., Çopur Doğrusöz M., Başaran U. 2020. Determination of silage quality of Narbon Vetch (*Vicia narbonensis* L.) and Ryegrass (*Lolium multiflorum* L.) mixtures. ÇOMÜ Ziraat Fakültesi Dergisi, 8(2), 391-396.
Oba M., Allen M.S. 1999. Evaluation of the importance of the digestibility of neutral detergent fiber from forage: Effects on dry matter intake and milk yield of dairy cows. *Journal of Dairy Science*, 82, 589-596.
Okoye C.O., Wang Y., Gao L., Wu Y., Li X., Sun J., Jiang J. 2023. The performance of lactic acid bacteria in silage production: A review of modern biotechnology for silage improvement. *Microbiological Research,* 266, 127212.
Oliveira A.S., Weinberg Z.G., Ogunade I.M., Cervantes A.A., Arriola K.G., Jiang Y., Adesogan A.T. 2017. Meta-analysis of effects of inoculation with homofermentative and facultative heterofermentative lactic acid bacteria on silage fermentation, aerobic stability, and the performance of dairy cows. *Journal of Dairy Science,* 100(6), 4587-4603.
Oliveira R.L., Ribeiro O.L., Bagaldo A.R., Borja M.S., Correia B.R. 2018. Nutritional composition and fermentative characteristics of Massai grass silage added with licuri (Syagrus coronata) cake. *Semina: Ciências Agrárias,* 39, 1189-1198.
Ozduven M.L., Kursun Onal Z., Koc F. 2010. The effects of bacterial inoculants and/or enzymes on the fermentation, aerobic stability and *in vitro* dry and organic matter digestibility characteristics of triticale silages. *Kafkas Universitesi Veteriner Fakültesi Dergisi*, 16(5), 751-756.
Özdüven M.L., Tepeli C., Okuyucu B. 2017. The effects of lactic acid bacterial inoculants on the fermentation and aerobic stability of sunflower silages. *Tekirdağ Ziraat Fakültesi Dergisi,* 14(2), 9-15.
Pang H., Tan Z., Qin G., Wang Y., Li Z., Jin Q., Cai Y. 2012. Phenotypic and phylogenetic analysis of lactic acid bacteria isolated from forage crops and grasses in the Tibetan Plateau. *The Journal of Microbiology,* 50(1), 63-71.
Pahlow G., Muck R.E., Driehuis F., Oude Elferink S.J.W.H., Spoelstra S.F. 2003. Microbiology of Ensiling. In: Buxton D.R., Muck R.E., Harrison J.H. (Eds),

Silage Science and Technology (31-93). Madison (WI): American Society of Agronomy.

Peng C., Sun W., Dong X., Zhao L., Hao J. 2021. Isolation, identification and utilization of lactic acid bacteria from silage in a warm and humid climate area. *Scientific Reports*, 11, 12586.

Polan C.E., Stieve D.E., Garrett J.L. 1998. Protein preservation and ruminal degradation of ensiled forage treated with heat, formic acid, ammonia, or microbial inoculant. *Journal of Dairy Science*, 81(3), 765-776.

Sniffen C.J., O'connor J.D., Van Soest P.J., Fox D.G., Russell J.B. 1992. A net carbohydrate and protein system for evaluating cattle diets: II. Carbohydrate and protein availability. *Journal of Animal Science*, 70(11), 3562-3577.

SPSS (IBM SPSS Statistics for Windows). 2012. v. 21.0., IBM Corp SPSS Inc., Armonk, NY.

Sullivan M.L., Hatfield R.D. 2006. Polyphenol oxidase and o-diphenols inhibit postharvest proteolysis in red clover and alfalfa. *Crop Science*, 46, 662-670.

Şahin A. 2019. The Effect of different levels of molasses addition to Italian grass (*Lolium multiflorum* cv. caramba) silage on silage quality. Master thesis, University of Hatay Mustafa Kemal, Hatay.

Şen G., Erol T., Kara K., Demirci M., Karslı M.A. 2022. The effect of microbial inoculants and molasses on quality and *in vitro* digestibility of silages prepared with different proportions of ryegrass and Hungarian vetch. *Turkish Journal of Veterinary and Animal Sciences*, 46, 629-637.

Tekin M., Kara K. 2020. The forage quality and the in vitro ruminal digestibility, gas production, organic acids, and some estimated digestion parameters of tomato herbage silage with molasses and barley. *Turkish Journal of Veterinary and Animal Sciences,* 44, 201-213.

Uğurlu S., Okuyucu B., Özdüven M.L. 2022. The effects of bacterial inoculants on the fermentation, aerobic stability and feed value of rye (*Secale cereale* L.) silages. *Turkish Journal of Agriculture Food Science and Technology*, 10(3), 426-433.

Van Soest P.J., Robertson J.D., Lewis B.A. 1991. Methods for dietary fiber, neutral detergent fiber, and, non-starch polysaccharides in relation to animal nutrition. *Journal of Dairy Science,* 74(10), 3583-3597.

Wang C., He L.W., Xing Y.Q., Zhou W., Yang F.Y., Chen X.Y., Zhang Q. 2019. Fermentation quality and microbial community of alfalfa and stylo silage mixed with Moringa oleifera leaves. *Bioresource Technology*, 284, 240-247.

Weinberg Z.G., Ashbell G., Yaira H., Azrieli A. 1995. The effect of cellulase and hemicellulase plus pectinase on the aerobic stability and fiber analysis of peas and wheat silages. *Animal Feed Science and Technology*, 55, 287-293.

Weiss W.P., Conrad H.R., Pierre N.R.S. 1992. A theoretically-based model for predicting total digestible nutrient values of forages and concentrates. *Animal Feed Science and Technology*, 39(1-2), 95-110.

Yin X., Zhao J., Wang S., Dong Z., Li J., Shao T. 2022. The effects of epiphytic microbiota and chemical composition of Italian ryegrass harvested at different growth stages on silage fermentation. *Journal of the Science of Food and Agriculture*, 103, 1385-1393.

Yin X., Long J., Zhao J., Wang S., Dong Z., Li J., Shao T. 2023. Effects of growth stage on Italian ryegrass (*Lolium multiflorum* Lam.) silage fermentation from microbiological perspective. *Chemical and Biological Technologies in Agriculture*, 10, 19, 1-14.

Zhao J., Dong Z., Li J., Chen L., Bail Y., Jia Y., Shao T. 2019. Effects of lactic acid bacteria and molasses on fermentation dynamics, structural and nonstructural carbohydrate composition and in vitro ruminal fermentation of rice straw silage. *Asian-Australasian Journal of Animal Science*, 32, 783-791.

Received 28 January 2026; accepted for publication 20 April 2026

Manuscript submission

By submitting a manuscript authors warrant that their contribution to the Journal is their original work, that it has not been published before, that it is not under consideration for publication elsewhere, and that its publication has been approved by all co-authors, if any, and tacitly or explicitly by the responsible authorities at the institution where the work was carried out.

Authors are exclusively responsible for the contents of their submissions, the validity of the experimental results and must make sure that they have permission from all involved parties to make the data public.

Authors wishing to include figures or text passages that have already been published elsewhere are required to obtain permission from the copyright holder(s) and to include evidence that such permission has been granted when submitting their papers. Any material received without such evidence will be assumed to originate from the authors.

Authors must make sure that all only contributors who have significantly contributed to the submission are listed as authors and, conversely, that all contributors who have significantly contributed to the submission are listed as authors.

The journal Biotechnology in Animal Husbandry publishes manuscripts in the categories of:

Original scientific paper – a paper containing previously unpublished results of own research;
Review paper – a paper containing an original, detailed and critical presentation of a research topic in which the author has made a certain scientific contribution that is visible on the basis of self-citations;
Short communication – an original scientific paper in full format, but of a smaller scope or preliminary character.

The manuscript should be prepared according to the Instruction for authors using the Article Template and submitted following the link https://istocar.bg.ac.rs/en/journal-biotechnology-in-animal-husbandry/#, or sent to the e-mail address biotechnology.izsvgmail.com.

Manuscripts are be pre-evaluated at the Editorial Office in order to check whether they meet the basic publishing requirements and quality standards. They are also screened for plagiarism.

Authors will be notified by email upon receiving their submission. Only those contributions which conform to the following instructions can be accepted for peer-review. Otherwise, the manuscripts shall be returned to the authors with observations, comments and annotations.

Manuscript preparation

Authors must follow the instructions for authors strictly, failing which the manuscripts would be rejected without review.

The journal publishes papers in English. Please check the paper you submit for publication in regard to the English grammar and spelling. British and American English are equally acceptable.
Names in Latin should be written in italics.
Use standardized abbreviations and SI units.

The manuscript should contain: title, full names of authors, ORCID of authors, affiliations of authors, Abstract, Keywords, manuscript text (Introduction, Material and Methods, Results and Discussion, Conclusion), Acknowledgment, Conflict of interest, References.
Tables and figures should be incorporated into the text of the manuscript.

The manuscript should be prepared in Microsoft Word for Windows, Paper size: Custom size, Width 17 cm, Height 24 cm; format (Portrait), normal spacing (Single Space). Margins: Top 2.0 cm, 2.0 cm Left, Bottom 2.0 cm, 2.0 cm Right, no pagination (see Article Template https://istocar.bg.ac.rs/en/journal-biotechnology-in-animal-husbandry/

Use font Times New Roman, size 11 (except where it is stated otherwise), single space, justify

Title - Times New Roman, font size 14, bold, capital letters, justify

Authors – Times New Roman, font size 12, bold, specify the full names and ORCID of all authors on the paper. Use 1, 2, … numbers in suffix to refer to addresses of authors, only in the case of different affiliations (institution)

Affiliations of authors – Times New Roman, font size 9, normal, under affiliations of authors should be mentioned e-mail of corresponding author and after that category of paper.

Title of paragraphs should be in Times New Roman 14 **bold**, except for **Abstract** and **Key words** where font size is 11 **bold**

Abstract: up to 250 words, Times New Roman, font size 11, justify. Abstract should contain a brief overview of the methods and the most important results of the work without giving reference.

Key words: not more than 6. The selection carried out by relying on widely accepted international source such as a list of keywords Web of Science.

Introduction – present the review of previous research and objective of the paper

Materials and Methods – includes experimental animals, experimental design, laboratory methods, statistical analyses. Provide an ethical statement on animal welfare if applicable.

Results and Discussion – present investigation results separately from discussion or together as one chapter or separately. Presentation of the results should be precise and without repetitions, and include the evaluation of significant differences and other parameters.

Text and titles of tables, figures and graphs, Times New Roman, font size 9.

Tables and figures should be numbered and with adequate title and legend. Tables should be prepared according to instruction for forming of tables in Office Word. Each column in table must have heading and, when necessary, abbreviations should be explained in the legend/footnote.

Conclusion – contains a general conclusion based on the research results in relation to the set objectives of the study.

Title - in Serbian language, Times New Roman 14 **bold**, is stated, followed by authors in Times New Roman 11 *italic*.

Summary – in Serbian language, 250 max words

Key words – in Serbian language, not more 6

Note: non-Serbian authors should provide Summary in English language that will be translated to Serbian by Editor's office

Acknowledgment – contains the source of funding, the code of the project within which the research was conducted, as well as the institutions or persons who contributed to the research and are not listed as authors.

Conflict of interest – contains reported conflicts of interest or, in the event that they are not reported, contains a statement: 'The authors declare that they have no conflict of interest″.

References – should be in alphabetical order. Titles in the language of the original. Use only the full name of the cited journal.

Examples:

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)

Laws and Regulations:

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.

Web references can be listed separately (at the end of reference list) or can be included in the reference list.

Citations in the text:

State the last/family name of the author and the year of publication:

Petrović (2020);
In the case of two authors - Petrović and Jovanović (2020);
In the case of more than two authors - Petrović et al. (2021*);*
In the case of multiple references, list chronologically - (Petrović, 2020; Petrović and Jovanović, 2020; Petrović et al., 2021)
In case of multiple references by the same author in one year - (Petrović et al., 2021a; Petrović et al., 2021b)

Editor's office