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THE YELLOW MEALWORM (*TENEBRIO MOLITOR* L.) IN ANIMAL NUTRITION: ADVANCES AND PROSPECTS FOR SUSTAINABLE LIVESTOCK PRODUCTION

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Review article

Abstract: *Tenebrio molitor* (TM) is one of the most promising insect species proposed as an alternative to conventional food and feed in the last years. This insect is interesting for its valuable nutritional profile, lower environmental impact compared to traditional livestock and its ability to valorise organic waste. Optimising production is essential for insects farmers and several factors including environmental parameters, rearing density, nutritional strategies are involved. These aspects are essential to maximize the growth and productivity of TM. For instance, supplementing the basal diet of TM larvae with additional sources of protein, carbohydrates and lipids proved to enhance nutritional composition and growth performance of larvae. Likewise, the right combination of temperature, relative humidity, and oxygen, strongly influences development time and reproductive success. Moreover, adopting high breeding density of beetles can increase the overall productivity, although it may reduce the productivity per female. This review also examines the processing methods of TM larvae and their inclusion, in various forms, in the diets of poultry, rabbits, and aquaculture species. The incorporation at proper level of TM larvae meal showed to supports growth performance, feed efficiency and quality of the final product without compromising animal health. However, further efforts are needed to make TM meal cost-competitive with conventional feed ingredients. Finally, the review examines one of the most abundant by-products of the TM industry, the frass, highlighting its potential applications as a sustainable fertilizer, its effects on common crops, and its possible use as biochar to support more sustainable agricultural practices and environmental management strategies.

Key words: insect protein, circular economy, optimisation, alternative feed ingredient, frass

Introduction

Over the last few years, insect farming has become a major topic of discussion regarding food and feed alternatives, as well as economic and environmental sustainability. Given the projected growth of the world population by 2050 (United Nations, 2019) and the decreasing availability and rising costs of conventional raw materials used in feed production, it is essential to identify new strategies for producing nutritious and sustainable food and feed that are viable from an environmental, economic, and social perspective. These strategies should focus on shortening supply chains, reducing waste, and minimizing environmental impact. Insects have been proposed as a viable protein alternative in human, livestock and fish (Van Huis et al., 2013). They are typically harvested from nature in tropical countries, but they can also be semi-domesticated to increase production or easily reared in confined industrial facilities ranging from small to industrial scale (Oonincx and Boer, 2012).

Among insect species, *Tenebrio molitor* (TM) was the first one approved by EFSA for human consumption. Commonly known as the yellow mealworm, TM is one of the most studied and produced insect species in Europe (Krzyzaniak et al., 2022). It belongs to the Tenebrionidae family (Coleoptera), and it is typically considered a pest of farinaceous materials, which constitute its primary food. The life cycle of the yellow mealworm comprises four distinct metamorphic stages: egg, larvae, pupa, and beetle. The entire cycle duration is influenced by environmental parameters such as temperature, humidity and population size lasting from 60 to 90 days under optimal conditions (Hardouin and Mahoux, 2003) up to one or two years under uncontrolled environment (Selaledi et al., 2019). Larvae are characterised by an elongated cylindrical shape, six legs behind the head and two short appendages at the abdomen’s tips. During the growth phase, which lasts around 60 days under controlled conditions, the larvae undergo several moults until reaching adult size characterized by an average weight of 0.2 g and a length of 25-35 mm (Aguilar-Miranda et al., 2002). If not utilised for consumption or processed, larvae go against a period of latency and a transformation into pupae, a free-living creature of yellow colour and 1 cm long. The stage of pupae typically lasts about 6 days under farming conditions (Cotton, 1927), culminating in the emergence of beetles.

TM is particularly interesting due to a high environmental sustainability and the nutritive composition. From an environmental sustainability perspective, the life cycle assessment of TM can be compared to conventional protein sources such as meat, milk, fish meal, and soy due to the high energy consumption required to maintain optimal temperatures for rearing (van Huis and Oonincx, 2017). Nevertheless, considering direct emission levels, insects proved to be more efficient than conventional livestock, with 100 times lower total greenhouse gases emission compared to the 18% of the livestock sector. TM exhibits significantly lower CO_2 equivalents per unit of edible proteins compared to broiler chickens and beef (Oonincx and Boer, 2012). As for emissions, the land use required for insect production is significantly lower compared to traditional raw materials used in feed formulations (Oonincx et al., 2010) and livestock farming. Producing 1 kg of edible protein from TM requires only 10% of the land needed for beef production (van Huis et al., 2013). Furthermore, insect farming uses significantly less water compared to traditional livestock (Dobermann et al., 2017; van Huis and Oonincx, 2017). Embracing the concept of circular economy, TM can be fed waste raw materials, effectively converting them into high-nutritional-value food through a bio-conversion process (van Huis, 2022). This represents a significant advantage in terms of valorisation, making it possible to use organic waste as a resource for producing valuable products rather than disposing of it.

Additionally, the nutritional composition of TM larvae closely resembles that of conventional meat products, positioning them as a promising alternative source of nutrients for both human and animal diets. TM larvae are particularly rich in protein, comprising about 50% of their dry matter (DM), and contain approximately 30% lipids, which are high in beneficial fatty acids FA such as oleic, linoleic, and palmitic acids (Kröncke et al., 2019; Trukhanova et al., 2022). TM larvae also supply most of the essential amino acids required daily and serve as an excellent source of minerals, except for calcium, though calcium levels can be enhanced through dietary modifications. Furthermore, they are a valuable source of vitamins, including vitamin B_{12} (Nowak et al., 2016).

Given these considerations, insect farming offers a promising solution to the growing demand for sustainable and efficient food and feed alternatives in the face of global population growth and environmental challenges. Their ability to thrive on waste materials further enhances their role in a circular economy, reducing competition for human food resources while valorising organic waste. Despite some challenges, such as the energy needed for rearing, the overall benefits of insect farming make it a compelling option for addressing food security, environmental sustainability, and economic viability.

Tenebrio molitor farming

Ensuring optimal environmental conditions is crucial in TM rearing to optimize and improve farm productivity. Temperatures commonly used in TM farming range from 25 to 28 °C (Koo et al., 2013; Kim et al., 2015). Within this range, the productive performances of both adults and larvae are maximized. Temperatures below 17 °C have been reported to inhibit embryonic development, while temperatures above 30 °C led to increased mortality mainly due to stress condition of the insects (Koo et al., 2013). Temperature also affects energy assimilation efficiency, with higher values observed at temperatures ranging from 23.3 to 31 °C. Additionally, the content of proteins and lipids in TM varies according to temperatures (Bjørge et al., 2018). Closely related to temperature, relative humidity (RH) represents a source of water for insects influencing their adsorption capacity and several factors during the entire life cycle. Optimal RH values ranging between 60 and 75% have been reported in previous research (Manojlovic, 1987; Punzo and Mutchmor, 1980). A combination of optimal temperature and humidity is necessary for oviposition, embryological development, and eggs hatching. Specifically, dry conditions with about 12% RH can cause embryo death due to water loss from the eggs (Punzo and Mutchmor, 1980). RH also influences the number and length of instars, adult female activity, and larval growth rates. Larval growth is maximized at RH values of 90-100% and reduced at values around 30% (Hardouin and Mahoux, 2003). However, excessively high humidity levels can create an optimal environment for the growth of undesirable pests such as mites. Water intake plays a crucial role in TM rearing. Despite TM is able to absorb water from the humidity of the environment, as reported above, levels of RH higher than 60% can reduce time for larvae growth. Additionally, optimal growth is achieved with the provision of a water source. For instance, offering fresh carrots to larvae increased their survival rate and reduced development time (Oonincx et al., 2015). Similarly, the combination of vegetables with milk enhanced larval growth rates, as observed in the study of Lee et al. (2024).

Photoperiod also has a significant impact on the growth of TM larvae. Despite this species being negatively phototropic, longer light exposure during farming appears to be beneficial for larval growth. Specifically, shorter larval development times and longer pupal periods have been observed under conditions of 14 hours of light and 10 hours of dark. Furthermore, long-day conditions have been reported to increase the eclosion rate and the pupation time (Kim et al., 2015). However, a previous study reported a higher survival and growth rate of larvae in condition of constant darkness at 25 °C and 30 °C (Eberle et al., 2022).

Another environmental parameter influencing the TM rearing is the availability of oxygen. Specifically, low oxygen concentrations of about 10-15% inhibit growth rates, cause developmental abnormalities in mealworm populations and increase larval mortality (Greenberg and Ar, 1996; Loudon, 1988). Conversely, hyperoxia conditions lead to a lower number of instars and reduced final larval biomass compared to normoxia (Greenberg and Ar, 1996).

Among the factors influencing the productivity and the reproductive success of TM, the breeding density is one of the most significant (Morales-Ramos et al., 2012). High breeding densities of larvae affect the number and the duration of larval moults, resulting in fewer larval instars and smaller larvae (Morales-Ramos et al., 2012; Morales-Ramos and Rojas, 2015). Weaver and McFarlane (1990) observed a faster larval growth at a density of 0.33 larvae/cm² compared to

0.03 larvae/cm², while larvae reared in isolation produced larger female pupae. Furthermore, in the study of Morales-Ramos and Rojas (2015), increasing the density from 0.44 larvae/cm² to 3.51 larvae/cm² reduced the growth, the feed consumption, and the feed efficiency of TM larvae. Concerning the breeding density of the beetles, high densities are typically linked to increased progeny production overall (Berggren et al., 2018; Deruytter et al., 2019; Zim et al., 2022; Palumbo et al., 2024) (Figure 1). However, when examining progeny production per individual beetles, females raised at lower densities are significantly more prolific than those raised at higher densities (Morales-Ramos et al., 2012; Berggren et al., 2018; Deruytter et al., 2019; Frooninckx et al., 2022; Palumbo et al., 2024) (Figure 2). Indeed, high breeding density causes crowding stress in insects, which reduces the reproductive capacity of beetles along with an increased mortality due to cannibalism (Berggreen et al., 2018; Gerber, 1984; Morales-Ramos et al., 2012). Previous research has shown that all Tenebrionidae, including TM adults, exhibit cannibalistic behaviour (Kuriwada et al., 2009; Morales-Ramos et al., 2012). Specifically, under mass-rearing conditions, high densities lead to increased cannibalism of eggs, particularly those near the surface of the substrate (Kuriwada et al., 2009; Morales-Ramos et al., 2012). To prevent eggs cannibalism, separating the eggs from the beetles is a viable alternative. This can be achieved by using an oviposition grid, which allows the ovipositor to pass through the holes in the grid and lay eggs deep in the substrate. This way, the adults are unable to reach and eat the eggs. Indeed, as observed by Froonickx et al. (2022), the use of an oviposition grid resulted in a significantly higher yield of eggs.

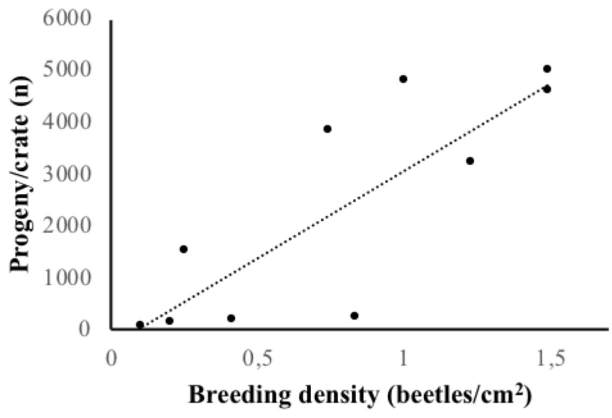

Figure 1. Linear regression of progeny produced per crate according to the beetles breeding density observed by Berggren et al. (2018), Deruytter et al. (2019) and Zim et al. (2022), modified.

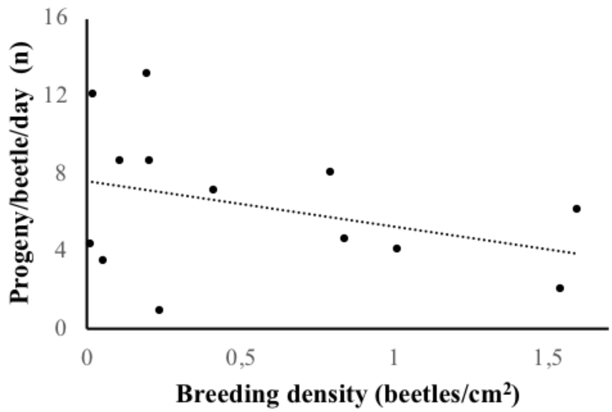

Figure 2. Linear regression of progeny produced daily per beetles according to the beetles breeding density observed by Morales-Ramos et al. (2012); Berggren et al. (2018), Deruytter et al. (2019) and Frooninckx et al. (2022), modified.

Tenebrio molitor feeding

In addition to environmental conditions, the productivity of TM can be influenced by several factors closely linked to farming techniques. One of the most significant is the choice of feeding substrate. Although TM can be raised exclusively on wheat bran, its diet can be supplemented with additional sources of protein, vitamins, minerals, and organic matter such as carrot, potato, and cabbage to provide essential nutrients. The quality of the feed directly affects oviposition: poor-quality diets can lead to fewer eggs laid and higher female mortality (Gerber and Sabourin, 1984). Van Broekhoven et al. (2015) found that incorporating 40% yeast-derived proteins into the diet of TM larvae shortened larval development time, reduced mortality, and increased weight gain and survival rates. However, this also affected the larvae's fatty acid (FA) profile, resulting in a comparable or slightly less favourable *n-6*/*n-3* ratio than a wheat bran-only diet. Feed intake significantly influences various aspects of TM life cycle. For example, supplementing wheat bran with protein sources at a 1:4 ratio has been shown to shorten development time and improve feed conversion efficiency and fecundity. Similarly, adding carbohydrate sources at the same ratio enhances feed utilization, growth, development time, survival, and beetle fecundity (Morales-Ramos et al., 2013). Including protein and carbohydrate sources at a 1:4 ratio with wheat bran also significantly reduces the number of larval instars and the total development time (Morales-Ramos et al., 2010). Furthermore, diets containing 33-39% protein can reduce the time to pupation (Oonincx et al., 2015). Increasing dietary protein by supplementing with pea flour and rice flour has also been shown to raise the protein content of the larvae and to increase their levels of essential amino acids (Kröncke et al., 2023). Additionally, Rumbos et al. (2020) demonstrated that incorporating specific amylaceous commodities with protein contents ranging from 11% to 14%, as well as milk-based feed and layer hens feed with protein contents of 62% on average, significantly increased the larval biomass produced. However, feeding TM larvae legume flour led to a reduction in larval body weight, despite the substrate's high protein content. This suggests that while higher dietary protein generally promotes faster insect growth, as Rumbos et al. (2020) noted, the use of legume flours had an inhibitory effect, indicating that other dietary components also play a crucial role in TM larval development.

Including lipids in the diet of TM has also been tested and demonstrated to have beneficial effects at low concentrations, while high concentrations can be detrimental. This is because high lipid levels can cause substrate agglomeration, reduce aeration and subsequently cause respiration problems in mealworms (Alves et al., 2016). The FA content of the diet seems to strongly influence the lipid profile of TM. For instance, Melis et al. (2019) found that rearing mealworms on brewer's spent grain, which is rich in polyunsaturated fatty acids (PUFA), increased the levels of *n-3* and *n-6* PUFA in the larvae and improved feed conversion ratio (FCR). Similarly, Lawal et al. (2021) showed that the supplementation of TM diet with 10% chia seeds increased the *n-3* FA favourably reducing the *n-6*/*n-3* ratio. In another study, Park et al. (2023) reported that including sesame cake in the TM diet enhanced the nutritional composition of the larvae. Regarding growth performance, the addition of 7.5% edible oils was found to slow larval growth (Rossi et al., 2022). In contrast, Ruschioni et al. (2020) found that including 25% olive pomace in the diet increased larval and pupal weights, improved survival rates, and extended development time. It also enhanced the protein and amino acid contents of the insects, without affecting their fatty acid profile. Furthermore, the inclusion of 5% and 10% camelina and linseed cake in the TM diet from four to nine weeks of age not only affected the FA profile of the larvae, reducing the *n-6*/*n-3* ratio, but also improved their growth performance by increasing the final body weight and reducing the FCR (Palumbo et al., 2025b).

Tenebrio molitor harvesting and processing

As previously reported, under controlled conditions, the life cycle of TM ranges from 60 to 90 days. Typically, larvae reach the appropriate stage for processing at 8 to 9 weeks of age, at which point they are separated from residual feed and frass before undergoing further processing. Ensuring the quality and the safety of insects and insect-based products is a significant challenge in industrial

TM production. TM fresh larvae typically exhibit a high microbial load, predominantly composed of gram-positive bacteria, including faecal and total coliforms (Belluco et al., 2013). Particularly, previous research showed the possibility to isolate Enterobacteriaceae and spore forming bacteria, not belonging to pathogenic species, from TM fresh larvae (Klunder et al., 2012). Insects processing includes killing, microbial decontamination by heat, drying and grinding (van Huis and Tomberlin, 2017). Thermal treatments such as boiling, blanching, drying and other cooking methods are commonly used for microbial inactivation to ensure the safety of insects-based products (Yan et al., 2023). Indeed, boiling TM larvae for 5 minutes was found to be an efficient process to inactivate Enterobacteriaceae, whereas boiling followed by refrigeration from 5 to 7 °C resulted efficient against spore forming bacteria and spoilage (Klunder et al., 2012). Boiling larvae at temperature ranging from 70-150 °C not only affect the microbial load but also ensure the inactivation of enzymes responsible of the browning reaction caused by the oxidation of phenolic compounds (Wang et al., 2017; Cacchiarelli et al., 2022). In addition to boiling, freezing is a commonly used practice able to stop the microbial growth guaranteeing a gentle and quick death for the insects, thereby reducing their suffering (Lenaerts et al., 2018). Prolonged killing times, in fact, should be avoided to reduce the suffering of the insects and to prevent FAs oxidation, which can occur due to the stimulated metabolism of energy reserves (Larouche et al., 2019). The killing method is a crucial stage as it can also influence the proximate composition (Adámková et al., 2017; Leni et al., 2019; Caligiani et al., 2019) and the sensory traits of the final product (Farina, 2017; Palumbo et al., 2025a). For instance, in *Hermetia illucens*, a reduction in FA content (Caligiani et al., 2019) and in certain amino acids (Leni et al., 2019) was observed when freezing was used as the killing method, compared to blanching. Conversely, Adámková et al. (2017) found that freezing best preserved the fat content of TM larvae.

Along with the killing method, the drying process significantly influences the quality of the final product. The application of high temperatures for extended periods are responsible of a visual impairment of the larvae due to pronounced darkening and shrinkage (Purschke et al., 2018; Trukhanova et al., 2022). Furthermore, high temperatures promote the degradation of proteins and vitamins, as well as the oxidation of lipids, which deteriorate the flavour and affect both technological and functional properties of the product (Bußler et al., 2016; Kröncke et al., 2018; Baek et al., 2019). Among the drying processes involving high temperatures, oven drying, and microwave drying are the most commonly applied and tested. These processes are often proposed as alternatives to freeze-drying due to long operating times and high cost of the latter. Freeze-drying preserves the texture, the nutritive value, the aroma, and the colour of the product, promoting a sublimation of water and avoiding the application of high temperatures (Huang & Zhang, 2012). Compared to freeze drying, microwave drying has been associated with a lower protein solubility (Kröncke et al., 2018). Additionally, microwave-treated larvae exhibited a reduction in vitamin B_{12} levels, and a darker colour compared to the fresh ones (Lenaerts et al., 2018). A dark brown colour was also observed in larvae dried using oven drying, along with significant shrinkage due to tissue collapse (Purschke et al., 2018). Thus, the optimal processing method is still not defined. However, a recent study reported that blanching at 100 °C for 180 s or freezing at -60 °C for 1h, followed by microwave drying at 103 °C for 15 min, preserved nutritional content, ensured microbiological safety, and was considered the preferred method for consumer acceptability (Palumbo et al., 2025a).

Before the use of TM larvae for protein extraction, lipid removal is required due to their high fat content. This step is essential not only to isolate the lipids for potential applications but also to enhance protein extraction, resulting in a more concentrated material with improved technological and functional properties (Choi et al., 2017). The solvents commonly employed for lipid extraction from TM larvae include ethanol, hexane-isopropanol mixture or hexane, all of which demonstrated high extraction yields (Bußler et al., 2016; Zhao et al., 2016; Purschke et al., 2017; Kim et al., 2020). However, an alternative and more sustainable method using supercritical CO_2 has shown an impressive extraction yield of 95% (Purschke et al., 2017). The protein extraction efficiency significantly influences the commercial production of food products rich in TM proteins. The chosen method should ensure a high extraction yield to meet large-scale production demands while preserving the functional properties of the proteins, resulting in a concentrate of protein that is as pure as possible (Zhao et al., 2016). The alkaline extraction followed by isoelectric precipitation is a commonly employed method for various substrates. In the case of TM protein extraction, this method resulted in an acceptable yield and a relatively high protein content of the extract (Bußler et al., 2016; Zhao et al., 2016; Kumar et al., 2019). Additionally, sonication has been observed as a further method that enhances protein extraction from edible insects (Choi et al., 2017).

Tenebrio molitor as feed

The nutritional composition of TM larvae closely resembles that of conventional meat products, positioning them as a promising alternative source of nutrients for both human and animal diets. TM have a high protein content, with crude protein comprising approximately 47-53% (DM), and a lipid content ranging 25-35% DM, depending on their diet and developmental stage. Their lipid profile includes a significant proportion of unsaturated fatty acids, notably oleic acid (C18:1) and linoleic acid (C18:2), as well as saturated FA such as palmitic acid (C16:0) (Kröncke et al., 2019; Trukhanova et al., 2022). They also supply most of the essential amino acids required daily and serve as an excellent source of minerals, except for calcium, though calcium levels can be enhanced through dietary modifications. Additionally, TM larvae are a valuable source of vitamins, including vitamin B_{12} (Nowak et al., 2016). The excellent nutritive composition is emphasised by the ease of breeding and the reduced environmental impact of TM production compared to livestock (van Huis et al., 2013).

For these reasons, TM larvae are not only a promising alternative to conventional feed sources but also a viable substitute for traditional fish and soybean meals used in feed formulations (Makkar et al., 2014). Fish meal is an ingredient extensively used in aquaculture, pigs, and poultry nutrition obtained from small wild-caught marine fish usually not suitable for human consumption. However, due to several factor including an excessive exploitation of wild fish stocks, the availability of fish meal is decreasing, and costs are increasing (FAO, 2016b; van Huis and Oonincx, 2017).

Poultry

Several authors have investigated the inclusion of TM meal in broiler chickens' diets and found no detrimental effects on growth performance or carcass traits at inclusion levels of up to 10%. Notably, the inclusion of 0.3% full-fat TM meal in broiler diets increased feed intake (FI) and body weight gain (BWG), thereby reducing the FCR (Benzertiha et al., 2020). Similarly, Sedgh-Gooya et al. (2020) reported an increase in BWG with a 5% inclusion level of full-fat TM meal, while no significant effects were observed on FI and FCR. Andrade et al. (2023) observed that a 2% inclusion of full-fat TM meal enhanced FI and weight gain in broiler chickens and also modulated the innate immune response. Furthermore, Biasato et al. (2016) and Elahi et al. (2020) found that full-fat TM meal inclusion levels of 7.5% and 8% had no significant impact on growth performance or carcass traits. In contrast, replacing the broiler diet with 5% and 10% of whole dried TM larvae was reported to positively influence BWG, carcass yield, meat composition, and welfare traits (Vasilopoulos et al., 2023). Higher inclusion levels of full-fat TM meal have been associated with negative effects on the caecal microbiota of broiler chickens, particularly at 10% and 15% inclusion levels (Biasato et al., 2019). Additionally, Bovera et al. (2016) reported that a 29.7% inclusion of full-fat TM meal reduced ileal digestibility of DM, organic matter, and crude protein. Moreover, TM larvae can also be offered live as environmental enrichment for broilers; Bellezza Oddon et al. (2021) found that a 5% inclusion of live larvae had no negative impact on growth performance or health status. In laying hens, the effects of TM meal supplementation have shown some variability. Rahmawati et al. (2022) reported that dietary supplementation with 2% and 5% full-fat TM meal enhanced the *n-3* content of eggs and increase egg weight, without significant effects on physical egg quality. However, Ait-Kaki et al. (2021) observed that a 5% inclusion level reduced both the final body weight of hens and egg weight, while shell breaking strength increased. Ko et al. (2020) found no significant effects on egg production with full-fat TM meal inclusion levels ranging from 1% to 3%; however, 3% inclusion level led to a more intense yolk colour and an increase in histidine content. Expanding beyond chickens, the inclusion of live TM larvae has been tested as a nutritional enrichment in laying quails. Dalle Zotte et al. (2024) reported that an inclusion of 10% did not compromise growth performances, indicating potential applications across various poultry species.

Rabbit

While the majority of research on TM as a feed ingredient has focused on poultry species, its potential applications extend to other livestock, including rabbits. In particular, incorporating 4% full fat TM meal into the diet of New Zealand White rabbits was found to increase final body weight (BW) and carcass yield (Kowalska et al., 2020). While the FA profile remained unchanged, a difference in amino acid content was observed, with higher levels of isoleucine and methionine in rabbit fed with TM meal. Similarly, in a subsequent study, Kowalska et al. (2021) reported that replacing 4% of soybean meal with full fat TM meal increased the rabbits' BW and muscle fat content, supporting the potential for partial replacement of soybean meal in rabbit diets. Additionally, the inclusion of 4% full fat TM meal was found to increase the apparent total tract digestibility of ether extract, acid detergent fibre, and acid detergent lignin, as well as to enhance the activity of caecal and colon bacterial N-acetyl-β-D-glucosaminidases (NAGases) (Strychalski et al., 2021). However, this inclusion level was associated with a reduction of enzyme activity and lower short-chain FA concentrations in the hindgut. The full replacement of soybean meal with 30 g/kg of TM meal has been shown to maintain rabbit performance without adverse effects (Volek et al., 2021). Furthermore, rabbits fed diets containing TM larvae meal exhibited reduced nitrogen losses in urine and decreased total nitrogen excretion. These findings were supported by a subsequent study, in which no adverse effects on growth performance or nitrogen output were observed (Volek et al., 2023).

Research on TM larvae fat inclusion in rabbit diets, primarily as a substitute for soybean oil, has also yielded promising results. Partial (50%) and total replacement of soybean oil with TM fat have been found to have no detrimental effects on growth performance, apparent digestibility, gut morphometric indices, or organ histopathology in rabbits. Moreover, this substitution did not negatively impact consumer acceptance of rabbit meat and, notably, reduced the meat's susceptibility to oxidation (Gasco et al., 2019).

Aquaculture

The inclusion of TM in aquaculture feed, either as a partial or complete replacement for fishmeal (FM), has been proposed as a promising strategy to enhance the sustainability of the aquafeed industry. Defatting TM meal is generally preferred, as it facilitates the extrusion process, resulting in more stable pellets and reduced degradation (Rema et al., 2019).

The replacement of FM with 15% or 30% of full fat TM meal was evaluated in the diet of rainbow trout (*Oncorhynchus mykiss*), showing no adverse effects on growth performance, protein utilization, or physiological status. However, at the highest level of FM replacement, a significant reduction in *n-3* FAs in the fillet was observed, along with a decreased viscerosomatic index and increased aerobic catabolism (Melenchón et al., 2020).

Studies on gilthead seabream (*Sparus aurata*) have shown promising results when partially replacing FM with TM larvae meal. Piccolo et al. (2014) reported that substituting 25% of FM with full-fat TM meal resulted in no significant negative effects on BWG or final BW. A subsequent study by the same research group (Piccolo et al., 2017) corroborated and extended these findings. They observed that 25% inclusion level of full-fat TM meal (corresponding to 35% of FM substitution on protein basis) led to statistically significant improvements in BWG, final BW, specific growth rate (SGR), and FCR.

Research on higher inclusion levels of TM meal in fish diets has yielded variable results across species. In rainbow trout, inclusion levels exceeding 25% of full-fat TM meal have been associated with adverse effects. FM replacement at 50%, 75%, and 100% with full-fat TM meal has resulted in significant reductions in BWG and final BW, coupled with increased FCR (Valipour et al., 2019). Additionally, a 50% inclusion of full-fat TM meal in replacement of FM has been shown to alter both total and free amino acid profiles in rainbow trout fillets, with notable reductions in free alanine, leucine, isoleucine, and lysine at the highest inclusion level (Iaconisi et al., 2019).

Referring to the European seabass (*Dicentrarchus labrax*), FM replacement of up to 80% with defatted TM meal has not compromised growth performance, nutrient utilization, flesh quality, or intestinal morphology (Basto et al., 2019). Even at 100% replacement of FM with defatted TM meal, growth performance parameters remained unaffected, although an increase in hepatosomatic index and alterations in FA profile were observed. At 80% replacement, protein and lipid digestibility remained unaltered along with growth performance (Basto et al., 2021).

In rainbow trout, complete replacement of FM with partially defatted TM meal has shown no significant adverse effects on gut and skin microbiota (Terova et al., 2021). High dietary inclusion of defatted TM meal, up to 100% replacement of FM, has been reported to increase lipid and energy content without significantly affecting blood gas pressure, electrolyte balance, or haematocrit (Sakhawat Hossain et al., 2025). These findings collectively suggest that the effects of TM meal inclusion are species-specific and dependent on the processing method of the meal (full-fat *vs* defatted). While high inclusion levels may be detrimental in some species, others appear to tolerate substantial FM replacement with TM meal without compromising key physiological parameters.

The inclusion of TM meal has also been investigated in Pacific white shrimp (*Litopenaeus vannamei*) diets, yielding promising results, albeit with some variability. Replacing up to 30% of FM with TM meal led to increased BWG, feed efficiency, and hepatopancreas index, whereas higher inclusion levels were associated with reductions in these parameters. Additionally, higher inclusion levels were found to decrease cholesterol, triglycerides, and glucose levels (Sharifinia et al., 2023). Contrasting results were reported by Chung et al. (2015), where FM replacement at 50% and 100% resulted in increased BWG and SGR, along with improved FCR.

The substitution of fish oil (FO) with TM oil in the diet of Pacific white shrimp has demonstrated varying effects depending on the level of replacement. While complete substitution of FO with TM oil did not significantly alter shrimp performance parameters (Panini et al., 2017), partial replacement has yielded more favourable outcomes. Eom et al. (2023) reported that replacing 50% to 75% of FO with TM oil resulted in enhanced feed utilization efficiency. Moreover, their findings suggest that the optimal FO replacement level is dependent on the desired outcome: a 50% substitution was found to be optimal for growth performance, whereas a 25% replacement showed the greatest benefit for disease resistance against *Vibrio parahaemolyticus*.

Tenebrio molitor by-products – frass

Frass is the primary by-product of large-scale insect farming. Although it is often considered waste, frass possesses a nutrient profile rich in readily mineralisable macronutrients (NPK) and micronutrients, making it suitable for various applications (Houben et al., 2021). Typical frass composition includes approximately 2-5% nitrogen, 1.5-2.6% phosphorus, and 1.1-2.0% potassium and about 14% lipids (Poveda et al., 2019; Houben et al., 2021; Antoniadis et al., 2023; Palumbo et al., 2024; Amorim et al., 2024).

Frass predominantly comprises insect excrement, along with smaller amounts of shed exoskeletons and undigested or residual feed. Utilisation of this by-product can enhance the sustainability and profitability of insect farms. Numerous studies have examined the diverse applications of frass, particularly its potential as an organic fertiliser in agriculture due to its favourable NPK composition. Its chemical profile closely resembles that of poultry manure, with comparable plant growth rates and yields observed (Hénault-Ethier et al., 2023).

TM frass, in particular, has gained attention as a promising organic soil amendment rich in both macro- and micronutrients as well as plant growth-promoting microorganisms (PGPMs) (Fuertes-Mendizábal et al., 2023; Nogalska et al., 2024). Research indicates that TM frass can match or even outperform synthetic fertilisers, especially when used in combination with mineral fertilisers. Application of TM frass at a concentration of 2% (v/v) to chard (*Beta vulgaris* var. cicla) has been shown to supply an NPK ratio of 3-1.5-2, along with essential micronutrients such as sulphur, calcium, magnesium, manganese, iron, and molybdenum. This treatment enhanced chlorophyll content, fresh weight, shoot length, and stem thickness (Poveda et al., 2019). The presence of calcium and magnesium in TM frass has been observed to mitigate the acidifying effects of urea when used in peat-based substrates (Nogalska et al., 2022).

TM frass, applied at 10 t/ha^{-1} (NPK: 5-2-2) has been found to increase barley biomass (Houben et al., 2021). In wheat cultivation, soils treated with TM frass, alone or in combination with mineral fertilisers, exhibited improved root development, grain quality, and yields, achieving results equal to or better than those obtained with conventional synthetic fertilisers (Nyanzira et al., 2023). The beneficial effects of TM frass as an organic fertiliser on crop growth are closely linked to its application rate. In lettuce cultivation, frass application at a concentration of 1% (v/v) supplied sufficient macronutrients to support the crop’s needs, resulting in increased aboveground biomass. However, when the application rate was raised to 2.5% (v/v), these benefits were reversed, leading to reduced plant growth, with the root system being particularly affected (Fuertes-Mendizábal et al., 2023).

TM frass can also be converted into biochar, offering promising applications in environmental remediation. Through pyrolysis, nutrients are stabilised, and a carbon-rich material is formed (Kumar et al., 2023; Thalassinos et al., 2023). Biochar derived from TM frass has been shown to function effectively as a bio-adsorbent for purifying wastewater and remediating contaminated soils (Yang et al., 2019). Moreover, TM frass biochar can aid in carbon sequestration supporting circular economy goals and reducing greenhouse gas emissions. TM frass biochar activated at various temperatures has been observed to capture 90% of CO_2 within 20 minutes, with biochar produced at 700 °C exhibiting the most favourable CO_2 storage and electrochemical behaviour (Wang et al., 2022).

Beyond these applications, recent research explored TM frass as bedding material for broiler chickens. Inclusion at 50-75% in replacement to wood shavings did not affect growth performance, while numerically reducing hock burn and footpad dermatitis incidence, and lowering microbial load, suggesting potential benefits for animal welfare (Tóth et al., 2025). A further study (Tóth et al., unpublished) with larger flocks is being conducted to confirm these preliminary results.

Conclusion

The optimisation of environmental parameters, including temperature, relative humidity, photoperiod, oxygen concentration, and rearing density, in conjunction with balanced nutritional strategies, is crucial for maximizing the growth and productivity of TM. While wheat bran serves as a suitable basal diet, supplementing with additional feedstuffs to the purpose of fulfilling the nutritional requirements in protein, carbohydrates, and lipids has been demonstrated to enhance larval development and nutritional composition. TM larvae represent a promising and nutrient-dense alternative to conventional feed ingredients across various livestock sectors, including poultry, rabbit, and aquaculture. When incorporated at appropriate inclusion levels, TM larvae meal supports growth performance and feed efficiency without compromising animal health. Furthermore, TM fat has shown potential as a viable substitute for traditional oils in feed formulations. However, the current limited commercial availability and relatively high production costs of TM larvae present significant challenges to their widespread adoption in livestock feed formulations. Competition with more economical conventional feed ingredients remains a substantial barrier to the large-scale implementation of TM in livestock nutrition. The by-product of TM production, the frass, is demonstrating significant potential as organic fertiliser and soil amendment. Its application could contribute to sustainable agricultural practices and environmental management strategies, potentially offering additional value streams for TM producers. Collectively, these attributes position TM and its associated by-products as potential contributors to sustainable, circular food production systems. However, continued research efforts are essential to further refine rearing methodologies, optimise nutrient profiles, and facilitate the sustainable scaling of production.

Žuti brašnari (*Tenebrio molitor* L.) u ishrani životinja: napredak i izgledi za održivu stočarsku proizvodnju

Antonella Dalle Zotte, Bianca Palumbo

Rezime

Tenebrio molitor (TM) je jedna od najperspektivnijih vrsta insekata predloženih kao alternativa konvencionalnoj hrani i stočnoj hrani poslednjih godina. Ovaj insekt je zanimljiv zbog svog vrednog nutritivnog profila, manjeg uticaja na životnu sredinu u poređenju sa tradicionalnim farmskim domaćim životinjama i svoje sposobnosti da valorizuje organski otpad. Optimizacija proizvodnje je neophodna za uzgajivače insekata i uključeno je nekoliko faktora, uključujući parametre životne sredine, gustinu uzgoja i strategije ishrane. Ovi aspekti su neophodni za maksimiziranje rasta i produktivnosti TM. Na primer, dopunjavanje bazalne ishrane larvi TM dodatnim izvorima proteina, ugljenih hidrata i lipida pokazalo se da poboljšava nutritivni sastav i performanse rasta larvi. Isto tako, prava kombinacija temperature, relativne vlažnosti i kiseonika snažno utiče na vreme razvoja i reproduktivni uspeh. Štaviše, usvajanje visoke gustine razmnožavanja buba može povećati ukupnu produktivnost, iako može smanjiti produktivnost po ženki. Ovaj pregled takođe ispituje metode obrade larvi TM i njihovo uključivanje, u različitim oblicima, u ishranu živine, zečeva i vrsta akvakulture. Pokazalo se da uključivanje brašna larvi TM na odgovarajućem nivou podržava performanse rasta, efikasnost ishrane i kvalitet finalnog proizvoda bez ugrožavanja zdravlja životinja. Međutim, potrebni su dalji napori kako bi se sačma od transkripcionih sastojaka učinila konkurentnom po ceni u odnosu na konvencionalne sastojke hrane za životinje. Konačno, pregled ispituje jedan od najzastupljenijih nusproizvoda transkripcione industrije, insektni izmet, ističući njegove potencijalne primene kao održivog đubriva, njegove efekte na uobičajene useve i njegovu moguću upotrebu kao biouglja za podršku održivijem poljoprivrednim praksama i strategijama upravljanja životnom sredinom.

Ključne reči: proteini insekata, cirkularna ekonomija, optimizacija, alternativni sastojak hrane za životinje, insektni izmet.

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Conflict of interest

The authors declare no conflict of interest.

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EFFECTS OF DIETARY SUPPLEMENTATION WITH *NIGELLA SATIVA* L. ON PRODUCTIVITY AND HEALTH IN DAIRY COWS

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Abstract: The aim of this study was to evaluate the effects of *Nigella sativa* seeds supplementation on the productive performances and health status of lactating dairy cows. The study was conducted at the Experimental Farm of the Research and Development Institute for Bovine Balotesti, Romania, between November and December 2023, on a number of 40 Romanian Black and Spotted dairy cows divided into two groups: the experimental group E (n=20 heads) and the control group C (n=20 heads). The experimental group received 20 g of *Nigella sativa* seeds/head/day for 28 consecutive days. Milk and blood samples were collected at day 0 and day 28. Statistical comparisons were performed using Student's t-test. Results revealed statistical differences for lymphocytes (P=0.0479), glucose (P=0.0473), uric acid (P=0.0222), and amylase (P=0.0032) in the experimental group (E) after the 28-days trial. *Nigella sativa* seeds, due to their bioactive and antioxidant constituents, demonstrated promising potential as a natural dietary additive for enhancing dairy cattle physiological stability.

Key words: *Nigella sativa*, dairy cows, productive performances, blood profile.

Introduction

Globally, there is growing interest in using medicinal plants as feed additives to enhance ruminant productivity and health. *Nigella sativa L.*, commonly known as black cumin, is an annual herb of the *Ranunculaceae* family native to Southwest Asia, the Mediterranean basin, and parts of Africa (Akram Khan and Afzal, 2016; Hannan et al., 2021). Its seeds have been traditionally used for both medicinal purposes and as a nutritional supplement in human and veterinary diets. *Nigella sativa* seeds contain approximately 26.7 % protein, 28.5 % fat, 24.9 % carbohydrates, 8.4 % crude fibre, and 4.8 % ash, along with appreciable levels of copper, phosphorus, zinc, iron, and provitamin A (Ahmad et al., 2013; Srinivasan, 2018). The biological activities of black cumin are largely attributed to its major bioactive compounds (Alberts et al., 2024): thymoquinone (antioxidant, anti-inflammatory, and anticancer properties), thymohydroquinone (a powerful acetylcholinesterase inhibitor), and thymol (virucidal effect). Due to its high nutritional value and content of polyunsaturated fatty acids (omega-6 and omega-3), phytochemicals, flavonoids, and saponins (Abbas et al., 2024), *Nigella sativa* can function as an antioxidant by inhibiting non-enzymatic lipid peroxidation, thus enhancing immunity and animal productivity (El-Naggar et al., 2018). These antioxidant properties are considered to be linked to a dimer of thymoquinone, respectively, nigellone (Abd El-Hafeez et al., 2014). Several studies have highlighted the positive effects of *Nigella sativa* supplementation in ruminant diets, including improved nutrient digestibility and milk production (Matthäus and Özcan, 2011; Obeidat, 2020; Shahbazi et al., 2022). Moreover, *in vitro* data from Medjekal et al. (2017) also suggest that *Nigella sativa* exhibits anti-methanogenic properties, with methane emissions reduced by 20%. Beyond its nutritive value, black cumin exhibits broad-spectrum antimicrobial, antifungal, antiviral, and antiparasitic activities (Forouzanfar et al., 2014), with efficacy against both Gram-negative and Gram-positive pathogens (Ishtiaq et al., 2013; Pandey et al., 2025), and can favourably modulate haematological and biochemical parameters in small ruminants (Zanouny et al., 2013).

This trial aims to evaluate the effects of *Nigella sativa* seeds supplementation on the productive performance and health status of lactating dairy cows, in order to enhance milk production efficiency and overall animal health.

Materials and Methods

The study was conducted at the Experimental Farm of the Research and Development Institute for Bovine Balotesti (44°36′46″N 26°4′43″E), Romania, between November-December 2023, following the practices and standards approved by Romanian Law no. 43/2014 and the Council Directive 2010/63/EU regarding the protection and handling of animals used for scientific purposes. A total of 40 multiparous Romanian Black and Spotted dairy cows were divided into two equal groups: the experimental group E (n=20 heads) and the control group C (n=20 heads). The experimental group received 20 g *Nigella sativa* seeds/head/day for 28 consecutive days. The *Nigella sativa* seeds were purchased from certified suppliers. No adaptation period was implemented before the start of the experiment. All animals were transitioned directly to the experimental diet on Day 1 of the study, ensuring uniform exposure to the dietary treatment from the outset. The cows that were included in the study were balanced for age, live weight, and milk yield (E group: 3.5 years old, 500-600 live weight, with an average of 21.96 kg of milk/head/day; C group: 4 years old, 500-600 live weight, with an average of

20.85 kg of milk/head/day). The cows were housed under tied stanchion barn conditions. All cows were fed twice daily, the diet/head/day consisting of 5 kg alfalfa hay, 30 kg corn silage, and 5 kg concentrates, salt, and water *ad libitum*. The chemical composition (dry matter, organic matter, crude protein, crude fat, crude fiber, nitrogen-free extract, and crude ash) of the feeds used in the diet of the dairy cows (alfalfa hay, corn silage, and concentrate) was determined using the classical WEENDE system of feed analysis. The chemical composition of black cumin (*Nigella sativa*) seeds was obtained from published literature sources (Ahmad et al., 2013; Srinivasan, 2018). The energy and protein values of the feeds and black cumin seeds were calculated based on the determined chemical composition and regression equations established, which are widely applied in Romanian nutritional research for ruminants (Stoica and Stoica, 2001). The dietary formulation was based on the nutritional requirements established by the National Research Council (2001), ensuring compliance with the recommended standards for lactating dairy cows based on their milk production and body weight. The nutritional value of the feeds and the composition of the diets used in the study are presented in Tables 1 and 2.

Table 1. The nutritional value of the feeds used in the study (g/kg forage)

Nutritive crude value	DM, kg	MNU	DIPN, g	DIPE, g	Ca, g	P, g
Alfalfa hay	0.87	0.64	93	75	10	1.9
Corn silage	0.26	0.21	13	17	1.2	0.5
Concentrates	0.90	0.98	123	85	9.7	6.5
***Nigella sativa* seeds**	0.94	0.96	95	74	10	5

DM=dry matter; MNU=milk nutrition units; DIPN=digestible intestine protein allowed by the nitrogen content of the fodder; DIPE=digestible intestine protein allowed by the energy content of the fodder; Ca=calcium; P=phosphorus.

Table 2. The diet used in dairy cows feed during the study

Feed	Kg	15-18	13-15	1200-1350	1200-1350	90	54-60
		DM, kg	**MNU**	**DIPN, g**	**DIPE, g**	**Ca, g**	**P, g**
Alfalfa hay	5	4.35	3.2	465	375	50	9.5
Corn silage	30	7.8	6.3	390	510	36	15
Basic ratio input	**35.00**	**12.15**	**9.5**	**855**	**885**	**86**	**24.5**
Concentrates	5.0	4.5	4.9	615	425	48.5	32.5
Total (C)	**40.00**	**16.65**	**14.4**	**1.470**	**1.310**	**134.5**	**57**
Nigella sativa	0.02	0.01	0.01	1.9	1.48	0.20	0.10
Total (E + C)	**40.02**	**16.66**	**14.41**	**1.471,9**	**1.311,48**	**134.7**	**57.1**

C=diet supplemented with 0 g *Nigella sativa* seeds/had/day; E=diet supplemented with 20 g *Nigella sativa* seeds yeast/had/day; DM=dry matter; MNU=milk nutrition units; DIPN=digestible intestine protein allowed by the nitrogen content of the fodder; DIPE=digestible intestine protein allowed by the energy content of the fodder; Ca=calcium; P=phosphorus.

Cows were milked twice daily (5:00 AM and 5:00 PM), milk samples (30 ml) were collected during morning and evening milking, before feeding, and stored at 4°C until analysis. Milk quality assessments, including fat (%), protein (%), and lactose (%), were performed using the Ekomilk 120 ultrasonic analyzer. Milk and blood samples were collected at the start of the experiment (day 0) and the end of the experiment (day 28). Blood samples were collected from the mammary vein in vacutainer tubes with K_3EDTA for hemoleukogram (2 ml/tube, and chilled to +4 °C), and sterile vacutainer tubes (9 ml/tube, centrifuged at 6000 rpm, 8 minutes) for biochemical examination, at 2–4 hours post-morning feeding. For cortisol analysis, blood samples were clotted at room temperature for 30–60 minutes, then centrifuged at 3000 rpm (~1500 × g) for 15 minutes at 20–22 °C. Red blood cell (10^6/µl), hemoglobin (g/dl), hematocrit (%), platelets (10^3/µl), white blood cell (10^3/µl), and leukocyte differential count: lymphocytes (%), monocytes (%), total neutrophils (%), were performed using an automated analyser Abacus Junior Vet 5. Glucose (mg/dl), fructosamine (µmol/l), total proteins (g/dl), blood urea nitrogen (mg/dl), creatinine (mg/dl), uric acid (mg/dl), alanine aminotransferase (IU/L), gama-glutamyl transferase (IU/L), alkaline phosphatase (IU/L), and amylase (IU/L), were determined using the automated biochemical analyzer Spotchem EZ SP-4430. For serum cortisol analysis (ng/ml), ELISA kits (Cortisol ELISA Kit, DiaMetra) were used. Statistical analyses were carried out using Minitab Statistical Software (version 17). The data were expressed as mean ±standard error of the mean (SEM) and coefficient of variation (CV, %). To test the influence of *Nigella sativa* seeds on the productive performances and blood parameters studied, the Student t-test was used.

The statistical model was:

$$\boldsymbol{Y_{ijk} = \mu + T_i + e_{ijk}}$$

where:

$\mathbf{Y_{ijk}}$ = dependent variables
$\boldsymbol{\mu}$ = overall mean
$\mathbf{T_i}$ = fixed effect of treatment *i*
$\mathbf{e_{ijk}}$ = residual experimental errors
Differences were considered statistically significant at $P \leq 0.05$, and trends $P \leq 0.1$

Results and Discussion

The animals included in the study achieved an average daily intake ranging between 16.65–16.66 kg DM/head/day, 14.40–14.41 MNU, 1.470–1.471.9 g DIPN, 1.310–1.311.48 g DIPE, 134.5–134.7 g Ca, and 57–57.1 g P, values that fall within standard nutritional requirements relative to body weight and milk yield. The results regarding the effects of *Nigella sativa* seeds on milk yield and milk composition are presented in Table 3. Milk yield was not affected by the addition of *Nigella sativa* seeds (E: 20.32±0.86 kg/head/day vs. C: 19.03±1.14 kg/head/day, P=0.1907) over the 28-day trial (P>0.05). For fat percentage, the experimental group (3.92±0.34%) and the control group (3.65±0.16%) differed slightly, with a nonsignificant difference (P=0.2810). Milk protein levels were also similar at the start of the trial (4.09±0.07% vs. 3.95±0.11%) and remained comparable after 28 days (3.97±0.14% vs. 3.88±0.14%), with no significant changes (P=0.3333). Lactose percentage changed slightly at the end of the period (4.66±0.05% in the experimental group compared to 4.51±0.11% in the control group), without statistical significance (P=0.1606). Contrary to our findings, several studies that investigated the use of *Nigella sativa* seeds in the diets of ruminants (sheep, goats, and water-buffalo) have reported beneficial effects on the efficiency of feed utilization as a result of improved nutrient digestibility coefficients, as well as an improvement in milk production (Randa, 2007; Sanad, 2010; Zanouny et al., 2012; Odhaib et al., 2018). Moreover, Mohamed et al. (2003) demonstrated that dietary supplementation with 100 mg *Nigella sativa* seeds/kg of body weight increased the milk fat content in goats and sheep. In a study conducted by Safaa (2005), the use of 5 g *Nigella sativa* seeds/head/day led to a significant increase in milk fat concentration and milk protein concentration in lactating ewes. Likewise, Sanad (2010) found that using 50 mg or 100 mg *Nigella sativa* seeds/kg of body weight significantly increased the fat and protein concentrations in milk from lactating water buffalo. Furthermore, after longer intervals of administering 80 g *Nigella sativa* seed powder /head/day, Goswami et al. (2018) reported an increase in milk production in dairy cows. In contrast, El-Saadany et al. (2008) reported that the percentage of fat and the percentage of protein in milk did not increase significantly when the diet of lactating goats was supplemented with *Nigella sativa* seeds. We therefore assume that the shorter administration interval in our pilot study may have limited the full expression of *Nigella sativa's* physiological effects in the experimental group. In this study, *Nigella sativa* seeds were administered for 28 days at a dosage of 20 g seeds/head/day. While this dosage aligns with values reported in the literature, the relatively brief duration may have constrained the manifestation of its potential benefits. Previous studies have typically employed longer supplementation periods, ranging from 30 to 90 days, depending on species and experimental design. In this context, although the dosage used in our study falls within the recommended range (20–50 g/day), the shorter administration period may have been a limiting factor in capturing the full spectrum of *Nigella sativa's* beneficial effects on performance and health indicators in dairy cows.

Table 3. Effects of *Nigella sativa* seeds on milk yield and milk composition in dairy cows

Parameters	E group		C group		P
	X±SEM	CV	X±SEM	CV	
Milk yield, kg/head/day					
Start (0 day)	21.96±1.08	21.98	20.85±0.99	21.38	P=0.2141
Final (28 days)	20.32±0.85	18.73	19.03±1.14	26.82	P=0.1907
Fat, %					
Start (0 day)	4.64±0.14	13.84	4.66±0.16	15.78	P=0.4537
Final (28 days)	3.92±0.34	38.75	3.65±0.16	20.57	P=0.2810
Protein, %					
Start (0 day)	4.09±0.07	8.13	3.95±0.11	12.77	P=0.1513
Final (28 days)	3.97±0.14	16.15	3.88±0.14	16.25	P=0.3333
Lactose, %					
Start (0 day)	4.62±0.04	4.63	4.67±0.03	3.54	P=0.1991
Final (28 days)	4.66±0.05	5.72	4.51±0.11	11.48	P=0.1606

E=experimental group; C=control group.

The haematological parameters (red blood cells, hematocrit, white blood cells, monocytes, and neutrophils) were not influenced (P>0.05) by the addition of *Nigella sativa* seeds in the diet of the dairy cows (Table 4). Although the lymphocyte percentage in the experimental group remained stable at 54.19% throughout the study, the statistically significant difference (P = 0.0479) compared to the control group (59.26%) suggests that *Nigella sativa* seeds supplementation may have contributed to maintaining lower lymphocyte levels. However, since no intra-group change was observed, this effect may reflect pre-existing differences rather than a direct immunomodulatory impact. For the platelets, the experimental group had a significantly lower number of platelets (E: 255.65±11.16 10^3/µl) compared with the control group (C: 309.10±17.5 10^3/µl), with a significant P-value of 0.0090. After 28 days, although both groups showed lower counts compared to the start, the difference was statistically nonsignificant (E: 241.65±13.9 10^3/µl vs. C: 277.95±18.9 10^3/µl, P=0.0952). The early significant reduction in platelet count in the experimental group (E) could indicate an anti-inflammatory or immunomodulatory response, which may equilibrate with time. This finding is in line with previous studies that have reported beneficial changes in immune parameters following *Nigella sativa* supplementation in ruminants (Sadarman et al., 2021). For instance, Habeeb and El Tarabany (2012) have shown that supplementing diets with different levels of *Nigella sativa* seeds significantly increased the concentration of hemoglobin and leukocytes in lactating goats.

Table 4. Effects of *Nigella sativa* seeds on hematological parameters in lactating dairy cows

Parameters	E group		C group		P
	X±SEM	CV	X±SEM	CV	
Red blood cells, 10^6/μl					
Start (0 day)	6.69±0.11	7.85	6.76±0.14	9.72	P=0.3590
Final (28 days)	6.74±0.99	6.59	6.56±0.13	9.36	P=0.1570
Hemoglobin, g/dl					
Start (0 day)	10.16±0.15	6.67	9.76±0.22	19.16	*P=0.0616*
Final (28 days)	10.02±0.14	6.33	9.73±0.18	8.36	P=0.1567
Hematocrit, %					
Start (0 day)	28.39±0.41	6.56	27.83±0.59	9.48	P=0.1795
Final (28 days)	28.22±0.34	5.51	27.33±0.59	9.68	P=0.1215
Platelets, 10^3/μl					
Start (0 day)	255.65±11.6	20.24	309.10±17.5	25.37	**P=0.0090**
Final (28 days)	241.65±13.9	25.66	277.95±18.9	30.42	*P=0.0952*
Withe blood cells, 10^3/μl					
Start (0 day)	9.09±0.62	30.75	9.45±0.50	24.05	P=0.3245
Final (28 days)	9.30±0.42	20.81	9.13±0.39	19.38	P=0.3912
Lymphocytes, %					
Start (0 day)	54.60±1.98	15.20	58.21±2.36	19.30	P=0.1633
Final (28 days)	54.19±2.52	20.81	59.26±1.93	14.55	**P=0.0479**
Monocytes, %					
Start (0 day)	7.20±0.89	55.57	8.48±0.61	32.60	P=0.1141
Final (28 days)	5.71±0.89	70.05	5.12±0.94	82.82	P=0.6761
Neutrophils, %					
Start (0 day)	34.5±1.81	23.42	36.91±2.23	27.00	P=0.2339
Final (28 days)	36.87±2.10	23.64	39.67±1.64	19.90	P=0.1299

E=experimental group; C=control group.

The descriptive statistic for serum biochemical indicators is presented in Table 5. The glucose levels were similar between the two groups (E: 54.00±0.93 mg/dl vs. 55.2±0.72 mg/dl; P=0.1569) at day 0. After 28 days, a significant increase in glucose levels was found in the experimental group (56.25±1.59 mg/dL) compared with the control group (51.35±2.03 mg/dl; P=0.0473). This increase may point toward improved energy metabolism or altered insulin sensitivity associated with the supplementation. The fructosamine levels, which reflect average blood glucose concentration over the preceding 2-3 weeks (Shohat et al., 2021), did not differ significantly at either time point. Similarly, total protein levels remained stable between the groups, suggesting no adverse effects on protein metabolism. Blood urea nitrogen and creatinine are valuable markers for protein metabolism and renal function, respectively (Avila et al., 2025). In our study, the obtained values did not show significant differences (P>0.05) at the end of the trial. This could indicate that kidney function was maintained despite the dietary changes. For the uric acid, there was a significant difference at 28 days for the experimental group showing lower uric acid levels (E: 0.82±0.03 mg/dl vs. C: 0.92±0.02 mg/dl; P=0.0222).

Table 5. Effects of *Nigella sativa* seeds on biochemical parameters in lactating dairy cows

Parameters	E group		C group		P
	X±SEM	CV	X±SEM	CV	
Glucose, mg/dl					
Start (0 day)	54.00±0.93	7.74	55.2±0.72	5.84	P=0.1569
Final (28 days)	56.25±1.59	13.88	51.35±2.03	16.17	**P=0.0473**
Fructosamine, µmol/l					
Start (0 day)	210.6±2.06	4.37	208.5±4.49	9.63	P=0.3366
Final (28 days)	201.75±1.89	4.19	201.6±2.60	5.76	P=0.4823
Total-Protein, g/dl					
Start (0 day)	7.34±0.08	4.89	7.53±0.12	7.16	P=0.1355
Final (28 days)	7.13±0.07	4.39	7.11±0.09	6.00	P=0.4008
Urea Nitrogen, mg/dl					
Start (0 day)	6.65±0.35	24.04	6.35±0.36	25.69	P=0.2581
Final (28 days)	9.6±0.46	21.48	9.7±0.61	28.20	P=0.4526
Creatinine, mg/dl					
Start (0 day)	0.93±0.02	10.57	0.90±0.02	14.55	P=0.2301
Final (28 days)	1.06±0.02	9.87	1.05±0.02	9.52	P=0.3705
Uric acid, mg/dl					
Start (0 day)	0.76±0.01	7.68	0.77±0.02	11.75	P=0.3469
Final (28 days)	0.82±0.03	17.49	0.92±0.02	13.48	**P=0.0222**
ALT, IU/L					
Start (0 day)	64.5±2.56	17.76	69.15±3.42	22.12	P=0.1452
Final (28 days)	71.2±2.23	14.00	58.5±1.44	11.02	**P=0.0004**
GGT, IU/L					
Start (0 day)	17.95±1.30	32.46	16.00±1.17	32.57	P=0.1305
Final (28 days)	24.65±2.42	43.86	18.85±2.66	63.22	**P=0.0302**
ALP, IU/L					
Start (0 day)	56.8±3.78	29.80	54.35±3.79	31.18	P=0.2550
Final (28 days)	56.7±4.14	32.68	49.85±3.00	26.92	P=0.0747
Amylase, IU/L					
Start (0 day)	326.20±15	20.57	353.3±15.3	19.40	P=0.1134
Final (28 days)	312.55±12.1	17.24	261.4±11.5	19.67	**P=0.0032**
Cortisol, ng/ml					
Start (0 day)	27.07±8.58	141.74	29.71±6.46	97.22	P=0.3650
Final (28 days)	15.97±6.32	176.90	23.54±6.48	123.2	**P=**0.1998

E=experimental group; C=control group; ALT=alanine aminotransferase; GGT=gama-glutamyl transferase; ALP=alkaline phosphatase.

Lower uric acid levels could be associated with changes in purine metabolism or an enhanced antioxidant status (Zhang et al., 2022). ALT and ALP are liver enzymes that serve as markers used in the determination of liver function (Ojediran et al., 2024). In our study, at the end of the trial, the experimental group showed significantly higher ALT levels (E: 71.2±2.23 IU/L vs. C: 58.5±1.44 IU/L; P=0.0004). Similarly, GGT levels increased significantly in the experimental group at 28 days (24.65±2.42 IU/L vs. 18.85±2.66 IU/L; P=0.0302), while changes in ALP were not statistically significant. While elevated liver enzymes could be a sign of hepatic stress, within the physiological context, the increases in ALT and GGT might be part of an enhanced metabolic process attributed to the bioactive compounds in black cumin. Amylase levels significantly dropped in the experimental group at 28 days (312.55±12.1 IU/L vs. 261.4±11.5 IU/L; P=0.0032). This may indicate an effect on pancreatic enzyme activity or improved carbohydrate digestion efficiency. Cortisol levels, which are a stress indicator (Grelet et al., 2022), showed a reduction in the experimental group compared to the control group (E: 15.97±6.32 ng/ml vs. C: 23.54±6.48 ng/ml) without statistical significance (P=0.1998) at 28 days. This suggests that there might be a trend toward reduced stress levels under supplementation. In a trial conducted by Forslund et al. (2010), the average cortisol values were between 10 nmol/l and 30 nmol/l (3.62 ng/ml–10.87 ng/ml) in healthy dairy cows. Similarly, Nedic et al. (2017) reported serum cortisol values between 4 ng/ml and 26 ng/ml in Holstein dairy cows kept under intensive systems, limits being in accordance with the ones registered by us.

Conclusions

The dietary inclusion of *Nigella sativa* seeds in the lactating dairy cows' diet did not significantly affect milk yield or milk composition over the 28-day trial. Hematological assessments revealed that most blood parameters (red blood cells, hemoglobin, leukocytes, neutrophils, and monocytes) were not influenced by the inclusion of *Nigella sativa* seeds. However, a significant reduction in the lymphocyte percentage was observed in the E group, suggesting a potential immunomodulatory effect. Biochemical analyses showed significant differences in several metabolic markers, including increased glucose levels, liver enzyme activities (ALT and GGT), reduced amylase activity, and lower uric acid concentrations following supplementation. These changes indicate that *Nigella sativa* may influence energy metabolism, liver function, and enzymatic activity in a manner that could be reflective of adaptive metabolic responses to its bioactive components. Overall, *Nigella sativa* can be used as a dietary supplement in dairy cow nutrition without negatively impacting milk production or health. Future research should aim to determine the optimal dosage and evaluate the long-term effects of *Nigella sativa* supplementation in dairy cattle, in order to fully realize its potential as a functional feed additive for improving health, productivity, and metabolic resilience in modern dairy production systems.

Efekti dodavanja u obrok crnog kumina (*Nigella sativa* L.) na produktivnost i zdravlje mlečnih krava

Marinela Enculescu, Stelian Sertu, Ioana Nicolae

Rezime

Cilj ove studije bio je da se proceni efekat dodavanja u obrok semena crnog kumina (*Nigella sativa*) na produktivne performanse i zdravstveno stanje mlečnih krava u laktaciji. Studija je sprovedena na eksperimentalnoj farmi Instituta za istraživanje i razvoj goveda Balotešti, Rumunija, između novembra i decembra 2023. godine, na 40 rumunskih crno-šarenih mlečnih krava podeljenih u dve grupe: eksperimentalna grupa E (n=20 grla) i kontrolna grupa C (n=20 grla). Eksperimentalna grupa je primala 20 g semena crnog kima/grlo/dan tokom 28 uzastopnih dana. Uzorci mleka i krvi su prikupljeni 0. i 28. dana. Statistička poređenja su izvršena korišćenjem Studentovog t-testa. Rezultati su pokazali statističke razlike za limfocite (P=0,0479), glukozu (P=0,0473), mokraćnu kiselinu (P=0,0222) i amilazu (P=0,0032) u eksperimentalnoj grupi (E) nakon 28-dnevnog ispitivanja. Seme crnog kumina (*Nigella sativa*), zbog svojih bioaktivnih i antioksidativnih sastojaka, pokazalo je obećavajući potencijal kao prirodni dodatak ishrani za poboljšanje fiziološke stabilnosti mlečnih krava.

Ključne reči: *Nigella sativa*, mlečne krave, produktivne performanse, krvni profil.

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

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GENETIC CHARACTERISATION OF KRIVOVIRSKA PRAMENKA SHEEP

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Abstract: One of strategic goals in the field of improving our sheep breeding is to preserve sheep genofund. An important aspect in that context is genetic characterisation of our locally adapted breeds. The present research was conducted in summer season on 30 Krivovirska Pramenka sheep aged about 3 years on average and raised in 3 different localities. The blood of these animals was taken by venepuncture of *v.jugularis* into 5 ml test-tubes that were properly marked and samples stored at 4°C in a refrigerator and transported to a laboratory. Isolation of total genomic DNA was performed by a commercial kit for the isolation of DNA from blood for which 100 μl blood per animal was used. For genotyping a set of 12 nucleus microsatellites recommended by the International Society for Animal Genetics – ISAG was used. Molecular data obtained by amplifying nucleus microsatellites were processed by means of following standard bioinformatics packages: GenAlEx 6.5 (Peakall and Smouse 2012), HP-Rare 1.0 (Kalinowski 2005), Coancestry v.1.0.1.9 (Wang 2011) and STRUCTURE 2.3.4. (Pritchard et al. 2000). On the basis of the research conducted a relatively high level of genetic diversity was determined in studied population as well as a small number of animal pairs whose level of relatedness was higher than expected one. At the same time, a low and positive inbreeding coefficient not statistically significant was detected what indicates that examined population of Krivovirska Pramenka sheep is not burdened by inbreeding. The results obtained are informative and with an application of additional markers can serve for obtaining the guideliness for preserving and sustainable breeding of Krivovirska Pramenka sheep in Serbia.

Key words: Krivovirska Pramenka, genotyping, microsatellites, diversity

Introduction

Biodiversity (Greek *bios* - life, *diversio* - diversity) means genetic, species-wide ecosystem and molecular diversity (Campbell, 2003). Survival of humans is connected with a preservation of an overall biodiversity because an extinction of certain species may devastate our ecosystems, lead to their degradation and deterioration while an irreversible loss of biological diversity can permanently affect the survival of the other species and therefore humans as well. Due to its geographical position, climatic conditions, zones and orobiom Serbia is an important centre of biodiversity both at a national, European and global level. It is one of 153 centers of the world biodiversity and one of 6 centers of European diversity (Strategy of Biological Diversity of the Republic of Serbia, 2011-2018). On the other hand, it is expected that by 2050 human population rise to 9 billion people. Because of that an agricultural production will represent an unprecedented challenge in the next 30 years. About 40 species of domestic animals and poultry contribute to meeting the human population needs in providing meat, milk, eggs, animal skin and manure. Among these species about 8 800 breeds and strains make animal genetic resources (AnGR) that represent a key component of biological diversity and at the same time are crucially important for food and agriculture (FAO, 2021). A significant segment of social and economic structures of rural communities which contribute to satisfying the human population needs in food (Tempelman and Cardelino, 2007) are sheep genetic resources what is particularly noticeable in the conditions of climatic changes taking into account that autochthonous sheep breeds function better in an extreme conditions of environment compared to other ruminants (Al-Dawood, 2017). However, depopulation and deagrarianisation of rural regions have caused the endangerment of sheep genetic resources regarding their biological survival (Odjakova et al., 2023).

In order to preserve such sheep breeds the first necessary step would be to make out a sheep morphological characterisation followed closely by a genetic one taking into account that in a small autochthonous population endangered in its biological survival there may occur a phenomena of inbreeding, genetic drift and decreased flow of genes what may reduce a genetic variability and lead to genetic erosion resulting in a loss of potentially useful alleles in population genetic fund and in a decrease of heterozygosity in population individuals. In Serbia this could, among other things, be a consequence of prevailing morphological characterisation not followed by a genetic one although it is now deemed a common procedure both in Europe and the world at large since it can quantify negative genetic processes that represent a threat to survival of endangered populations.

Over a considerable period of time, besides morphological characterisation of breeds, a genetic characterisation has also been performed globally by means of molecular markers (Baumung et al., 2004; Toro et al., 2009; FAO 2011; Lenstra et al. 2012) serving not only as an extra addition to morphological characterisation but being also of a considerable use to solving doubts regarding differentation between one or more breeds when on the basis of relatively small differences in phenotype it cannot be clearly determined. This has recently been seen on the example of Pramenka sheep when its local populations from the regions of Croatia and Slovenia have been characterised as separate breeds primarily on the basis of their unique genetic profiles (Ćinkulov et al., 2008; Report, 2021; Programme of Rural Development, 2014).

The most common type of molecular markers having been more then 30 years successfully used in genotyping in numerous animal species are nucleus microsatellites (Ellegren, 2004; Selkoe and Toonen, 2006; Guichoux et al., 2011; Abdul-Muneer, 2014; Putman and Carbone, 2014; Ellegren and Galtier, 2016). For genotyping selectively neutral microsatellites are used. On the other hand, variability of control region (hypervariable region – HVS or D-loop) mitochondrial genome (mtDNA) inherited through mother is very successfully used in the research of evolutionary history both of animal and human species as well as in the process of domestication (Beja-Pereira et al., 2004; Pedrosa et al., 2005; Wang et al., 2007; Achilli et al., 2009; Lancioni et al., 2013). Keeping in mind a long tradition of sheep breeding not only in the region of Serbia but also in the region of a whole Balkan Peninsula (Ciani et al., 2020) as well as an important geographical position of this area where in the course of history many migrations happened it can be expected that local sheep populations therein contain such mitochondrial maternal lines that can contribute to better understanding of evolutive history of sheep in a given region as well as in a process of domestication such as was the case during the analysis of variability of mitochondrial genome in donkey (Stanišić et al., 2017).

A poor research interest for Pramenka sheep raised in the territory of Serbia during past decades has resulted in only a small number of scientific studies on its various populations including Krivovirska Pramenka sheep as one of the less studied genotypes among its examined phenotypes.

According to data of Domestic Animal Diversity Information System (DAD-IS) for 2025 in Serbia a 2661 female and 74 male animals of Krivovirska Pramenka sheep are being bred. An effective size of population is 315 animals and that designates it as a potentially endangered sheep breed. In the research conducted by Ružić Muslić et al. (2023) the results of morphometrical characterisation and the indices of physical development in Krivovirska Pramenka sheep were presented. This study focuses on further research on this issue, i.e. genetic characterisation of Krivovirska Pramenka sheep in order to both prevent its genetic erosion and contribute to its preservation, scientifically based sustainable utilisation and conservation as an essential activity in ANGr management planning at local, national and global level. In addition, preservation of its genetic integrity is being closely connected with determination of an exact number of sheep breeds both in our region and in the world (FAO, 2000).

Material and Method

The research conducted in a summer season included 30 Krivovirska Pramenka individuals about 3 years old on average and raised in 3 different localities in Serbia: Valakonje, Podgorac and Boljevac.

Respecting all regulations regarding animal welfare and the consent granted by the Ethical Commission the animal blood samples were taken by venipuncture of *v.jugularis* into 5 ml test tubes properly marked and stored at 4°C in refrigerator and later transported to laboratory. The blood with EDTA in test tubes was frozen for further use. Isolation of total genomic DNA was performed by use of commercial kit for DNA extraction from sheep blood using 100 μl blood per animal. A set of 12 nucleus microsatellites recommended by the International Society for Animal Genetics – ISAG) and the Food and Agriculture Organisation of the United Nations – FAO for sheep genotyping was used for genotyping and 4 markers recommended by FAO 2011: INRA063, MAF65, MAF214 and OarFCB20 (FAO, 2011), and 8 markers from the updated ISAG list of markers recommended for sheep genotyping in 2017: CSRD247, ETH152/D5S2, INRA005, INRA006, INRA023, INRA172, MCM042 and MCM527.

However, during the optimisation of protocol for PCR amplification it was observed that amplification of locuses ETH152/D5S2, INRA005 and INRA023 was not successful in neither of the individuals even after repeated reactions of PCR amplifications. Besides, PCR amplification of locuses MAF214, INRA172 and MCM527 was only partly successful in studied sample. Thus, only 6 of 12 molecular markers selected for genotyping (CSRD247, INRA006, INRA063, MAF65, MCM042 and OarFCB20) provided reliable molecular and genetic data while molecular markers ETH152/D5S2, INRA005, INRA023, INRA172, MAF214 and MCM527 were determined as not suitable to be used in further analyses.

Taking into account that reliability of the results of molecular and genetic analyses depends on the number of used locuses it was decided that the analyses should be directed to an additional set of 12 nucleus microsatelites such as follows: BM1818, BM2113, ETH225, ILST29, ILST87, INRABERN185, MAF70, OarFCB48, SPS113, SRCRSP8, TCRVB6 and TGLA53, among which 8 were successfully amplified: ETH225, ILST87, MAF70, OarFCB48, SPS113, SRCRSP8, TCRVB6 and TGLA53. Therefore, genotyping of 30 Krivovirska sheep was done by using not 12 but 14 highly informative and reliable nucleus microsatelites. The list of markers is shown in Table 1.

Table 1. The list of nucleus microsatellites used in genotyping of Krivovirska Pramenka sheep population

	Marker
1	CSRD247
2	INRA005
3	INRA006
4	INRA23
5	INRA063
6	INRA172
7	MAF065
8	McM527
9	ETH152
10	MAF214
11	McM042
12	OarFCB20

An amplification of control region of mitochondrial genome about 1300 based pairs long being multiplied in all animals was done by means of the 15346for, 157rev and 15393 for primers (Lancioni et al., 2013) and F_BDG (Pedrosa et al., 2005).

Direct primers for multiplying nucleus microsatellites were marked by one of 4 fluorescence colours from DS-33 set of colours (Applied Biosystems). Nucleus loci were multiplied by a polymerase chain reaction (*Polymerase Chain Reaction* - PCR) using Type-it microsatellite kit (Qiagen) which enables a parallel amplification of more loci in one reaction. The amplification of control region of mitochondrial genome was done by standard PCR reactions. Separating the products of PCR amplification of nucleus microsatellites was done by a capillary electrophoresis (fragment analysis) using ABI 3130 Genetic Analyzer automatic sequencer (Applied Biosystems). Determination of the length of PCR amplification of nucleus microsatellites products was performed using a GeneMapper programme (Applied Biosystems) by comparing the length of the fragments of PCR amplification with a LIZ500 scale (ThermoFisher Scientific). Determination of primer sequence of nucleotids of control region of mitochondrial genome was performed by use of a commercial Sanger sequencing by a Macrogen Europe company (https://dna.macrogen-europe.com/eng/). Molecular data obtained by amplifying nucleus microsatellites in Krivovirska Pramenka sheep were processed by means of following standard bioinformatics packages: GenAlEx 6.5 (Peakall and Smouse, 2012), HP-Rare 1.0 (Kalinowski, 2005), Coancestry v.1.0.1.9 (Wang, 2011) and STRUCTURE 2.3.4. (Pritchard et al., 2000). Following standard parameters of genetic diversity: number of alleles (A), number of private alleles (PA), average number of alleles (Na), effective number of alleles (Ne), obtained heterozygosity (HO), expected heterozygosity (He) and coefficient of inbreeding

(F) were determined by using a GenAlEx 6.5 programme package. The abundance of alleles was determined by means of a HP-Rare 1.0 programme package. The values of parameters relatedness in animals (relatedness - r) and inbreeding coefficient of individual animals (F) were determined by use of TrioML estimator implemented in Coancestry v.1.0.1.9 programme package. An optimal number of genetic groups in mentioned population was determined by STRUCTURE 2.3.4 programme package.

Results and Discussion

Table 2 presents the results obtained for genetic characterisation of Krivovirska Pramenka sheep implying the parameters of genetic diversity. In the population of 30 Krivovirska sheep individuals a total of 98 alleles was detected on 14 studied loci. Number of alleles per locus (Na) ranged from 3 (ETH225) to 11 (ILST87 and MAF70) with an average count of 7 alleles per locus. An average effective number of alleles (Ne) was 4.24 while the values of this parameter per individual loci ranged from 2.36 (MCM042) to 6.55 (MAF70). The results obtained correspond with the research conducted by Odjakova et al. (2023), on 3 autochthonous sheep breeds (Rhodopean Tsigai, RT; Middle Rhodopean sheep, MRS; and Karakachan sheep, KS) in which they determined 4.72 effective alleles on average.

The average value of the abundance of alleles parameter performed according to the method of rarefaction was 7.00 for 60 copies of genes.

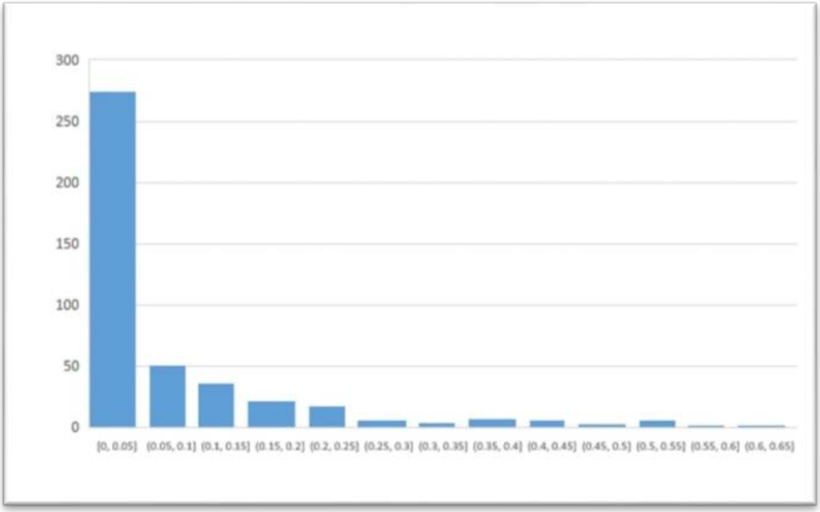
The heterozygosity obtained (Ho) per individual loci ranged from 0.433 (SPS113) to 0.900 (TCRVB6). The average obtained heterozygosity was 0.717 (SE=0.033). The expected heterozygosity (He) per individual loci ranged from 0.576 (MCM042) to 0.847 (MAF70). The mean expected heterozygosity was 0.737 (SE=0.024). The expected heterozygosity (He) that is being considered as a significant indicator of genetic diversity in Krivovirska pramenka population is somewhat lower than 0.76, 0.77, 0.77 values obtained in the studies by Odjakova et al. (2023), Georgieva et al. (2013), as well as Hristova et al. (2017), respectively. In the examined population a low and positive inbreeding coefficient of FIS=0.027 (SE=0.034) was detected but it was not statistically significant.

Table 2. Parameters of genetic diversity in examined population of Krivovirska sheep

Locus	N	A	Ne	Ar_{60}	Ho	He	F
CSRD247	30	7	5.33	7.00	0.767	0.812	0.056
ETH225	30	3	2.60	3.00	0.533	0.616	0.134
ILST87	30	11	6.23	11.00	0.800	0.839	0.047
INRA006	30	5	3.79	5.00	0.700	0.736	0.049
INRA063	30	6	2.85	6.00	0.667	0.649	-0.027
MCM042	30	5	2.36	5.00	0.667	0.576	-0.157
MAF65	30	7	3.16	7.00	0.733	0.683	-0.073
MAF70	30	11	6.55	11.00	0.833	0.847	0.016
OarFCB20	30	8	5.73	8.00	0.767	0.826	0.071
OarFCB48	30	8	5.83	8.00	0.833	0.828	-0.006
SPS113	30	5	3.03	5.00	0.433	0.670	0.353
SRCRSP8	30	8	3.94	8.00	0.667	0.746	0.106
TCRVB6	30	7	4.81	7.00	0.900	0.792	-0.136
TGLA53	30	7	3.23	7.00	0.733	0.690	-0.063
TOTAL/AVERAGE	30	98	4.24	7.00	0.717 (SE=0.033)	0.737 (SE=0.024)	0.027 (SE=0.034)

N – size of population; A – number of alleles; Ne – effective number of alleles; Ar_{60} – abundance of alleles per method of rarefaction for 60 gene copies; Ho – obtained heterozygosity; He – expected heterozygosity; F – inbreeding coefficient.

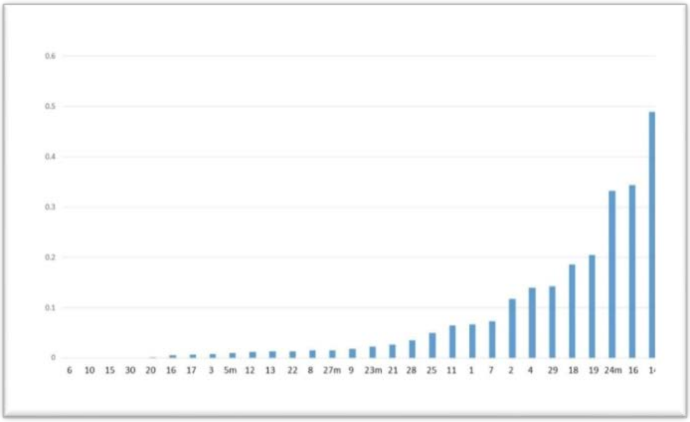
The values of the animal pairs relatedness parameter (relatedness - r) that can give an insight into a pedigree of studied animals and the inbreeding coefficient of individual animals determined by use of TrioML estimator are shown in graphs 1 and 2, respectively.



Graph 1. The values of relatedness–r parameters in animal pairs determined by TrioML estimators

Relatedness of the pairs of animals in a studied population of Krivovirska sheep ranged from 0.00 to 0.622 in the highest number of animal pairs the relatedness ranging from 0.0 to 0.05 was observed implying that unrelated animals are in question. However, the pairs whose value of r - parameter was ≥0.50 (KV14/KV25, KV3/KV11, KV11/KV17, KV13/KV29, KV9/KV25, KV16/KV23m, KV2/KV16, KV12/KV27m, KV14/KV26 and KV23m/KV28) and the two pairs of animals whose value of the same parameter was ≥0.60 (KV19/KV27m and KV25/KV26) were detected. Taking into account that ~0.5 value of r-parameter indicate the first-degree relatives it can be concluded that in the studied population the pairs of animals being in a higher degree of relatedness than expected are present as well. For the purpose of determining the pedigree of studied animals it is however necessary to perform a calibration of the value of r parametre using the animal with known pedigree (Wang, 2011).

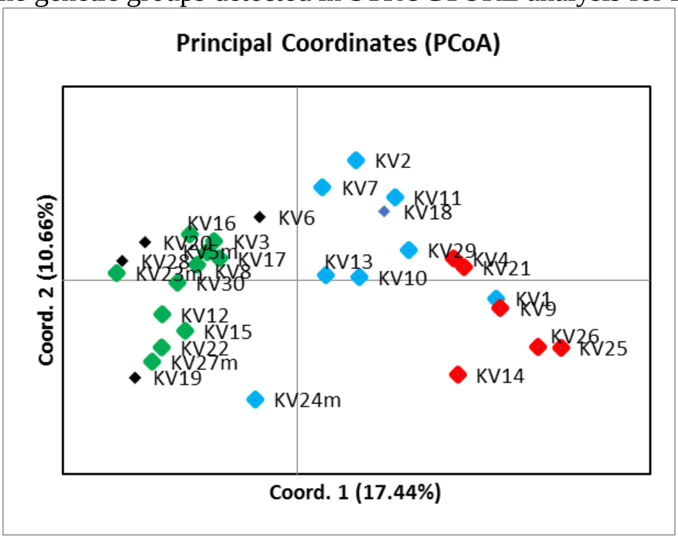
The inbreeding coefficient in individual animals (Graph 2) ranged from 0.00 (KV_6, KV_10, KV_15 and KV_30) to 0.49 (KV_14) not exceeding the value of 0.25 in majority of animals suggesting that the inbreeding is not present in a high degree in majority of animals.



Graph 2. The values of parameters of inbreeding coefficients in individual animals of Krivovirska Pramenka sheep

PCoA analysis performed on the basis of genetic distances of the animal pairs is shown in Graph 3.

In PCoA analysis the first and second coordinates explain 17.44% and 10.66% of a molecular variability, respectively. The first and second coordinates cummulatively explain 40.90% molecular variability. Distribution of the scores of animals in a two-dimensional space defined by the first and second coordinates most match the genetic groups detected in STRUCTURE analysis for K=3.

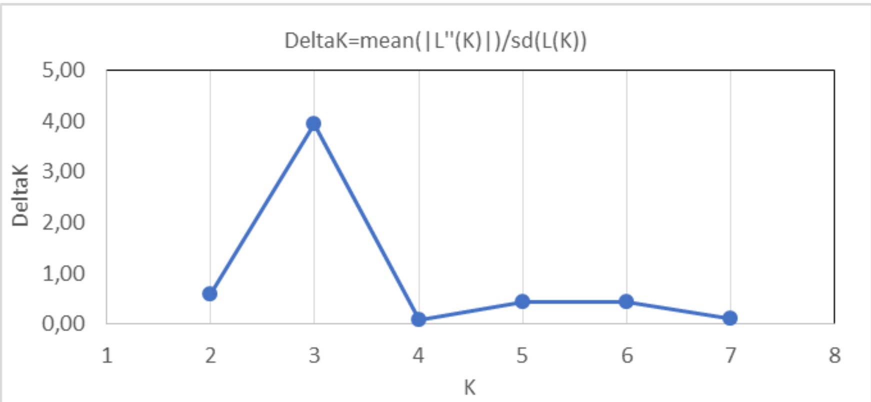


Graph 3. PCoA analysis performed on the basis of genetic distances of the animal pairs (animals characterised with a high degree of relatedness distributed either in red, blue or green gene pool in STRUCTURE analysis are marked by a rhomb of a corresponding colour while the other animals are marked by a black rhomb).

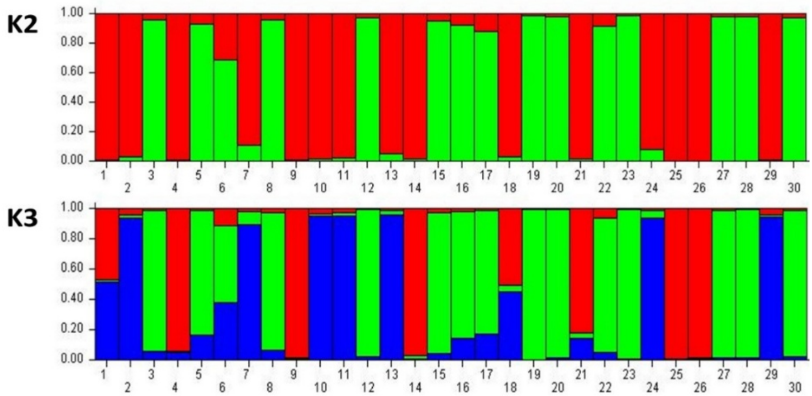
A substructure of studied population including 30 individuals of Krivovirska strain was determined by Bayesian method implemented in a STRUCTURE 2.3.4. bioinformatics package incorporating already given parameters: MCMC pre-analysis of 20 000 iterations, MCMC analysis of 20 000 iterations, the correlated allele frequency model and the relatedness of individuals per admixture model. The number of assumed genetic groups was K=1-8 while the number of analysis repetitions for each group was 10.

The optimal number of genetic groups, determined by an application of ΔK Evanno model (Evanno et al., 2005) was 3 (Graph 4). For K=3 a certain number of animals having a high share of relatedness (qi) distributed in each of 3 gene pools named by colour for easier tracking as red, green and blue gene pools was observed.

A display of substructure of 30 Krivovirska sheep studied population based on the results of STRUCTURE analysis for the number of K2 and K3 genetic groups is presented in Picture 1. Apart from the animals with a high share of relatedness (qi) distributed in one of the three gene pools the animals in which the admixture of gene pools was observed were also detected.



Graph 4. The optimal number of genetic groups in studied sample



Picture 1. Substructure of studied population of 30 Krivovirska sheep individuals based on the results of STRUCTURE analysis for the number of K2 and K3 genetic groups.

Three genetic groups detected in STRUCTURE analysis are matching the separation of animals obtained in PCoA analysis (Graph 3). Namely, per first PCoA coordinate the animals distributed in the so-called red and blue gene pool are separated from the animals distributed in the so-called green gene pool while per second PCoA coordinate the animals distributed in red gene pool are mostly separated from the animals distributed in a blue gene pool.

In spite of a small number of genotyped animals the results obtained in present research can offer useful information regarding both the structure of Krivovirska Pramenka sheep population and the estimation of genetic diversity serving as a starting point in designing effective breeding programmes.

Conclusion

Based on the research conducted it can be concluded that a relatively high level of genetic diversity was determined in the examined population of 30 Krivovirska sheep from the region of Serbia as well as a small number of animal pairs with the relatedness level higher than expected.

In the analysed population a low and positive inbreeding coefficient not statistically significant was detected indicating that the examined population of Krivovirska sheep is not burdened by inbreeding.

The obtained genetic data can be deemed both reliable and informative and together with morphological and other data and analysis on more populations as well as the application of additional markers in analyses can help formulate the guidelines and strategies for planned conservation of Krivovirska sheep in Serbia.

Genetska karakterizacija krivovirske pramenke

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Rezime

Jedan od strateških ciljeva na polju unapređenja ovčarstva je očuvanje genofonda ovaca. Važan aspekt u tom kontekstu je genetska karakterizacija naših lokalno adaptiranih rasa. Istraživanje je sprovedeno na 30 grla krivovirske pramenke, prosečne starosti oko 3 godine, u letnjoj sezoni, gajenih na 3 različita lokaliteta. Od navedenih životinja, uzeta je krv venepunkcijom *v.jugularis,* u epruvete od 5 ml, koje su uredno obeležene i uzorci su uskladišteni na 4°C u hladnjaku, nakon čega su transportovani u laboratoriju. Izolacija totalne genomske DNK je obavljena primenom komercijalnog kita za izolaciju DNK iz krvi za šta je korišćeno 100 μl krvi po grlu. Za genotipizaciju je korišćen set od 12 jedarnih mikrosatelita koji su preporučeni od strane Međunarodne organizacija za animalnu genetiku (*International Society for Animal Genetics* – ISAG). Molekularni podaci dobijeni umnožavanjem jedarnih mikrosatelita su obrađeni primenom standardnih bioinformatičkih paketa: GenAlEx 6.5, HP-Rare 1.0, Coancestry v.1.0.1.9 i STRUCTURE 2.3.4. Na osnovu obavljenih istraživanja, utvrđen je relativno visok nivo genetičkog diverziteta u ispitivanoj populaciji kao i mali broj parova grla kod kojih je stepen srodstva viši od očekivanog. Istovremeno, detektovan je nizak i pozitivan koeficijent inbridinga koji nije bio statistički značajan što ukazuje na to da ispitivana populacija krivovirske ovce nije opterećena inbridingom. Dobijeni podaci su informativni i uz primenu dodatnih markera mogu poslužiti za dobijanje smernica za očuvanje i održivo gajenje krivovirske rase ovaca u Srbiji.

Ključne reči: Krivovirska pramenka, genotipizacija, mikrosateliti, diverzitet

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

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MILK PRODUCTION TRAITS OF SIMMENTAL COWS IMPORTED FROM AUSTRIA AND THEIR DAUGHTERS REARED IN SERBIA

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Abstract: The objective of this study was to examine the milk traits of Simmental cows imported from Austria and their offspring born on the P.D. “DMN” farm, located in the municipality of Požarevac in Serbia and to determine the statistical significance of the differences between the milk production results of mothers and daughters. After Germany, Austria is the second largest exporter of pregnant Simmental heifers in both Europe and worldwide. The research included a total of 409 cows with 572 lactations recorded from the beginning of the farm’s operation in 2019 until the end of 2024. Production differences among animals in standard lactations were analyzed, along with comparisons of standard lactation results between mothers and daughters. By comparing the production results between mother cows and their daughters in the first and second standard lactations, a statistical significant difference in milk yield was found ($p<0.001$), while in the third lactation, the differences were not statistically significant ($p>0.05$). All the animals included in the research originated from the same farm, which enabled a precise comparison between mothers and daughters due to the combined influence of the farm and other environmental factors during the exploitation period.

Key words: dairy cows, milk production, milk traits, cattle import

Introduction

The production of cow’s milk is primarily of biological importance (for raising offspring), as well as of economic importance (in the food industry for the global population). Consequently, the total milk yield represents the most significant milk trait, determined both for the entire lactation period and for the standard lactation lasting 305 days. This standardization enables a reliable comparison of lactations of different durations.

The decrease in the number of livestock, the agricultural population, and the number of farms has been a decades-long trend in the Republic of Serbia. Aleksić et al. (2009) reported that the state of livestock production in Serbia is unfavorable, as the number of all types of domestic animals and poultry has been constantly decreasing. The largest decline in livestock numbers has been recorded since the early 2000s across all types of domestic animals.

Bogdanović et al. (2012) stated that approximately 59% of dairy farms in Serbia have up to 20 hectares of arable agricultural land available, with an average of about 10 hectares. On the other hand, about 55% of farms rear up to 15 head of cattle, with an average of 6 head per farm. Over 86% of surveyed farmers expressed an intention to expand existing production by increasing the number of animals, mainly through imports and stricter selection of parent pairs, as well as by improving nutrition, housing, and care conditions (Bogdanović et al., 2012). Sredojević et al. (2025) recommend that the rationalization of total milk production costs, based on proper planning of the quantity, quality, composition, and price of animal feed, should be improved in order to make farms economically profitable and to enable the economic sustainability of milk production. Given the limited capacity of animal organisms to adapt to the rapid pace of climate change, sustainable milk production remains a significant challenge in the context of global climate change (Mićić et al., 2025).

A large number of Simmental cattle have been imported from Germany and Austria over time, resulting in the Simmental breed comprising about 80% of the total cattle population in the country. Additionally, there has recently been a declining trend in the number of farmers keeping 1–3 dairy cows in Serbia (Perišić et al., 2012; Pantelić et al., 2021).

Popović (2014) cites data from the Statistical Office of the Republic of Serbia, based on the 2012 agricultural census, which indicates a total of 908,102 head of cattle in Serbia. However, at the beginning of 2025, according to the veterinary database, the total number of cattle was 714,730 head (https://cas.vet.minpolj.gov.rs/). Subić and Vasiljević (2022) state that despite favorable natural conditions for improved livestock production in the Republic of Serbia, the livestock sector is mostly characterized by a downward trend in the number of animals in the most important livestock categories. Between 2010 and 2019, the total number of cattle decreased annually at a rate of 0.43%.

The Republic of Serbia is among the European countries where the Simmental breed of cattle is well adapted and has been the most predominant breed for over a century. It has been used to improve domestic autochthonous cattle breeds, resulting in a significant replacement or crossbreeding of local cattle populations with the Simmental breed. The breed has primarily spread through the importation of females, breeding stock, and breeding bull semen from Austria, Switzerland, and Germany. Based on the current status of Simmental cattle breeding in the Republic of Serbia, as well as organizational, technical, and economic capacities in livestock production, and in accordance with basic genetic principles, the breeding objective for a controlled population of the Simmental breed-with a maximum of 25% dairy breed genes-defines specific milk traits. These include a milk yield of over 5,500 kg per standard lactation, a minimum milk fat content of 4.00%, a minimum protein content of 3.40%, a protein-to-fat ratio of 1:1.15-1.20, an age at first insemination of 15-18 months, and an age at first calving of 24-27 months (MBP, 2019).

Freyer et al. (2008) state that the antagonism between the continuous increase in milk production and the decrease in productive lifespan, accompanied by declining fertility in cows, is an ongoing and growing problem in the global cattle industry. The same authors further suggest that crossbreeding dairy cattle breeds can be an effective strategy to improve the functional traits of heifers and cows.

Kučević et al. (2005) observed the most important milk yield traits of cows and their daughters from the same sires of the Simmental breed in Germany and Serbia. They reported a significantly greater absolute difference in milk production of +1,057 kg in the primiparous cow generation in Germany, with differences at a high level of significance ($p \leq 0.05$). A statistically highly significant difference ($p \leq 0.01$) was also found for milk fat quantity and milk fat content, with daughters tested in Germany showing higher production.

Ajili et al. (2007) report that cows with the highest milk production in early lactation, unlike those with average production, are utilized more intensively and are culled from the herd at a higher rate.

Stanojević et al. (2022) report results from their research indicating a milk yield of 5,520 kg in standard lactation, with a milk fat content of 3.94%.

The Simmental (Fleckvieh) breed accounts for 74.7% of the total cattle population in Austria, with a population of 1,397,682 head according to 2021 data. It is reared from the Alpine mountain regions to the lowlands in the east. In 2021, Austria had 313,658 cows bred on 12,020 farms, of which 73% operate under difficult working conditions. In 2023, milk production control was performed on 268,760 cows. Over a 305-day lactation, an average of 7,910 kg of milk was produced, containing 4.17% milk fat and 3.37% protein, totaling approximately 600 kg of fat and protein combined. Among 71,960 first-calving cows, an average production of 7,187 kg of milk with 4.16% milk fat and 3.37% protein was recorded (https://www.fleckvieh.at/zucht/fleckvieh).

The breeding program in Austria is based on genomic selection and aims to quickly and efficiently achieve the breeding objectives. The targeted use of young bulls shortens the generation interval and enables faster and greater genetic progress in traits with low heritability.

The genomic selection program includes the selection of approximately 60 young bulls from around 4,000 candidates each year (selection ratio of 1:60). From these genomically selected bulls, the best six are annually chosen based on progeny testing of their offspring. Regarding insemination proportions, the goal is that 75% of registered cows in Austria be inseminated by young bulls, while for bull dams the target proportion is 90%. If applied consistently, this breeding program can achieve approximately 10% genetic progress per year, with a tendency toward greater robustness (https://www.genetic-austria.at/en/fleckvieh-simmental/fleckvieh-info-13571.html). Thanks to these efforts, Austria is one of the largest exporters of genetic material, with more than 23,000 Simmental cattle exported annually. Simmental genetic material from Austria is also sold as bull semen or in the form of frozen embryos (https://www.fleckvieh.at/zucht/fleckvieh/). Thus, pregnant heifers imported to Serbia, along with their genetic background, influence the improvement of selection effects even after calving through their female offspring. Furthermore, the study presents the production data of the first three lactations of mothers and daughters under Serbian production conditions.

Materials and Methods

Material – Farm

The research for this study was conducted at the dairy farm of the agricultural holding "DMN" Veliko Crniće, located in the municipality of Požarevac, Serbia (44°33′13″ N, 21°17′05″ E). Both mothers and daughters achieved milk production in their own first three lactations on the same farm under the same conditions of management, feeding plan and technological procedures.

The farm was reconstructed in 2018, when 130 pregnant Simmental heifers were imported from Austria. In 2019, an additional 60 cows were imported, followed by another 30 cows in 2022. Currently, the farm has close to 600 cows of all categories, with plans to reach 600 cows by 2026 and 1,000 cows including their progeny thereafter.

All animals are kept in a free-stall system, distributed by category across six facilities. The buildings are oriented in an east-west direction to align with the prevailing wind. The sides are not fully walled, allowing for good ventilation and natural lighting. Additionally, the buildings are equipped with wind protection nets used during adverse weather conditions.

Feed for all animals on the farm is prepared as a Total Mixed Ration (TMR) tailored separately for each category. The advantages of TMR include increased dry matter intake, leading to higher milk production and improved average daily gain in calves. Additionally, TMR promotes optimal rumen fermentation by providing a consistent diet with every bite, preventing sudden and frequent stresses on rumen microflora and eliminating selective feeding.

Milking is performed twice daily, with the milk collected in a 6,000-liter milk collector (Lactofreeze). The cows are divided into five groups based on production and stage of lactation: the first group includes cows producing more than 30 liters; the second, 25-30 liters; the third, 20-25 liters; the fourth group consists of cows in the pre drying period; and the fifth group includes dried-off cows. The third facility has a capacity of 80 stalls and uses a milking robot for cow milking. The milk is transported to a dedicated milk collector with a capacity of 4,000 liters. Cows have access to the milking robot up to three times within 24 hours. Through the software, various milking parameters can be monitored individually for each cow, as well as for each quarter of the udder.

Method – Data processing

During data collection, a total of 572 completed lactations up to August 2024 were analyzed. Production results were determined through regular milk yield control (AT4 control). Milk yield data of cows are recorded daily. By entering the number of a cow, all relevant data can be accessed, including daily milk yield, cumulative milk yield from the beginning of lactation until a specific day, and data from all previous lactations. In addition to these, data related to veterinary interventions-such as insemination dates, calving dates, treatments, and therapies-are also recorded.

The milk yield control analyzed the following milk yield properties:

- Days of lactation (days)
- Milk yield in standard lactation (kg)
- Milk fat yield in standard lactation (kg)
- Milk fat content in standard lactation (%)
- Milk protein yield in standard lactation (kg)
- Milk protein content in standard lactation (%)

To examine differences in milk production between mothers and daughters, as well as across individual lactations, the T-test procedure in the SAS statistical package was used (SAS Institute Inc., 2011). Descriptive statistics were calculated using the MEANS procedure, while the T-test procedure was employed to examine statistically significant differences between the analyzed animal groups within the same statistical package.

Results and Discussion

According to the main breeding program for Simmental cattle in Central Serbia (MBP, 2019), the breeding objective for a controlled population-with a maximum of 25% dairy breed genes-is defined as follows: a milk yield of over 5,500 kg in standard lactation, a minimum milk fat content of 4.00%, and a minimum milk protein content of 3.40%. In their research, Mićić et al. (2022) report an average daily milk fat content of 4.04% and a protein content of 3.23% for the examined population of cows in Central Serbia.

Table 1 compares the production results of cows from the first import and their daughters in the first standard lactation.

A statistically highly significant difference ($p<0.001$) was found in all examined production parameters, except for protein content in standard lactation, where a statistical highly significant difference was observed ($p<0.01$).The established milk production on the farm-over 8,000 kg for first-calving mothers and over 7,000 kg for first-calving daughters-is significantly higher than the production reported for individual producers in Central Serbia by Nikšić et al. (2011). Examining the production potential of Simmental heifers in Serbia, Nikšić et al. (2011) report an average milk yield of 4,348 kg with 3.93% milk fat and a milk fat yield of 171.1 kg, which is significantly lower than the cows in the present study. The milk production determined in standard lactation was also higher compared to the results reported by Stepić et al. (2018), who found an average yield of 6,659.9 kg of milk with 4.09% milk fat and 3.40% protein.

It is interesting to note (Čačić et al., 2022) that Simmental cows calving female calves in their first and second lactations had higher milk yield than those calving male calves, although the differences were not statistically significant. In the same study, the milk yield of first-lactation cows was recorded as 4,630.98 kg for mothers of male calves and 4,639.91 kg for mothers of female calves.

According to the report and results from the breeding program control in 2023 (Report, 2023), a sample of 14,824 first-calving cows in Central Serbia showed an average milk production of 4,973 kg in standard lactation, with 3.99% milk fat and 3.20% protein content. Considering these results, we can conclude that the animals examined in this study significantly exceed the average milk yield of the first-calving cows in Central Serbia. The milk yield determined in standard lactation was also higher compared to the results obtained by the aforementioned authors. Pantelić et al. (2013) report a significantly higher milk fat yield in first-calving Simmental cows in Serbia. Stanojević et al. (2022) report a milk yield of 5,052 kg with a milk fat content of 3.92% for first-calving cows.

Nikšić et al. (2023), examining both imported and domestic populations of Simmental cows, state that the origin of the cows has no statistically significant effect ($p>0.05$) on the milk fat content of first-lactation cows. However, housing conditions and their interaction with cow origin are statistically highly significant difference ($p\leq0.001$). The domestic cow population shows a fat content of 3.94%, while imported cows have a slightly lower milk fat content of 3.92%.

Table 1. Comparison of cows and their daughters in the first standard lactation

Traits	Mothers		Daughters		$t^{(p)}$
	Mean	Std.Error	Mean	Std.Error	
Milk yield in standard lactation (kg)	8143.1	117.6	7127.8	133.9	5.65^{***}
Milk fat content in standard lactation (%)	4.17	0.01	4.04	0.01	8.67^{***}
Milk fat yield in standard lactation (kg)	339.3	4.65	288.2	5.39	7.15^{***}
Protein content in standard lactation (%)	3.38	0.005	3.35	0.007	3.15^{**}
Protein yield in standard lactation (kg)	274.9	3.79	238.9	4.49	6.12^{***}
N	112		79		

*** ($p<0.001$), ** ($p<0.01$), * ($p<0.05$), ^{ns} ($p>0.05$)

Table 2 compares the production results of cows from the first import and their daughters in the second lactation. Milk yield traits in a standard 305-day lactation were examined. Statistically highly significant differences (p< 0.001) were found in all analyzed milk production parameters.

Table 2. Comparison of cows and their daughters in the second standard lactation

Traits	Mothers		Daughters		$t^{(p)}$
	Mean	Std.Error	Mean	Std.Error	
Milk yield in standard lactation (kg)	8633.3	123.1	7830.3	220.6	3.41^{***}
Milk fat content in standard lactation (%)	4.13	0.007	4.04	0.01	7.53^{***}
Milk fat yield in standard lactation (kg)	356.2	4.93	315.9	8.80	4.28^{***}
Protein content in standard lactation (%)	3.40	0.003	3.34	0.01	9.10^{***}
Protein yield in standard lactation (kg)	293.3	4.15	261.6	7.35	4.02^{***}
N	93		42		

*** ($p<0.001$), ** ($p<0.01$), * ($p<0.05$), ^{ns} ($p>0.05$)

Stanojević et al. (2022) report lower values for cows in the second lactation, as shown in Table 2, with an average milk yield of 5,553 kg and a milk fat content of 3.94%.

Table 3 compares the production results of cows from the first import and their daughters in the third lactation. No statistically significant difference (p>0.05) was found in milk yield in standard lactation trait. For the next four examined milk traits, statistically significant difference were found (p<0.001; p<0.05).

By comparing the milk yield in standard lactation between imported cows and their daughters across the first and second lactations, statistically highly significant difference were found (p<0.001), while in the third lactation, no statistically significant differences were observed (p>0.05). The lack of statistical significance may be related to the relatively small number of animals in the sample, which can increase the standard error. Therefore, increasing the sample size might lead to statistically significant results.

Table 3. Comparison of cows and their daughters in the third standard lactation

Traits	Mothers		Daughters		$t^{(p)}$
	Mean	Std.Error	Mean	Std.Error	
Milk yield in standard lactation (kg)	8926.1	174.4	8064.0	514.3	1.92^{ns}
Milk fat content in standard lactation (%)	4.08	0.008	4.01	0.02	3.66^{***}
Milk fat yield in standard lactation (kg)	363.8	6.78	323.3	21.02	2.29^{*}
Protein content in standard lactation (%)	3.41	0.004	3.32	0.01	8.14^{***}
Protein yield in standard lactation (kg)	304.0	5.85	267.4	16.96	2.44^{*}
N	69		14		

*** (p<0.001), ** (p<0.01), * (p<0.05), ^{ns} (p>0.05)

Stanojević et al. (2022) report a milk yield of 5.703 kg with 3.96% milk fat in cows completing their third lactation, while in this study, both mothers and daughters produced more than 8,000 kg of milk and more than 4.00% milk fat content.

For the trait milk fat content in standard lactation, across all observed lactations-first, second, and third-the differences between mothers and daughters were statistically highly significant difference (p<0.001). Although this study focuses on examining the milk production traits of mothers and their daughters, the potential effect on statistical significance may stem from differences in rearing conditions during the heifer phase. The mothers were raised as heifers in Austria before calving, whereas the daughters were raised on the farm in Serbia.

Conclusions

By comparing the milk production results in the first, second, and third lactations between imported cows and their daughters in Serbia, statistically significant differences were found in the examined production parameters, except for milk yield in the third standard lactation, where no statistically significant difference was recorded. This result of the comparison in the third lactation should be considered with caution given the very small sample size and large statistical error. The obtained results can serve as a basis for further research related to the importance of heifer rearing and its correlation with milk production traits, but also with other important traits from the breeding goals that effect on milk production (for example, type traits, age at first insemination, body condition).

Based on the analysis of completed lactations of Simmental cows on the studied farm, it is evident that the milk yield in a standard lactation is well above the national average in the Republic of Serbia. When compared with results from other authors, a higher milk yield in standard lactation was observed, while the milk fat and protein contents are consistent with levels reported by other studies. If other farms were to improve their production conditions to the level of those on the studied farm, the average milk production in Serbia would likely be higher.

Osobine mlečnosti krava simentalske rase uvezenih iz Austrije i njihovih ćerki odgajenih u Srbiji

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Rezime

Cilj ovog rada je bio da se ispitaju proizvodni rezultati krava simentalske rase uvezenih iz Austrije i njihovog potomstva rođenog na farmi P.D. "DMN" koja se nalazi u opštini Požarevac. Posle Nemačke, Austrija je drugi najveći izvoznik steonih junica simentalske rase u Evropi i svetu. Istraživanjem je obuhvaćeno ukupno 409 krava koje su ostvarile 572 laktacije u periodu od početka rada farme 2019. godine do kraja 2024. godine za osobine mlečnosti u standardnoj laktaciji. Upoređene su razlike u proizvodnji između grla u standardnim laktacijama i upoređivani su rezultati proizvodnje majka – ćerka u standardnim laktacijama. Upoređivanjem rezultata proizvodnje između krava majki i njihovih ćerki u prvoj i drugoj standardnoj laktaciji utvrđena je statistički značajna razlika ($p<0,001$) u prinosu mleka, dok u trećoj laktaciji razlike nisu bile statistički značajne ($p>0,05$). Sva grla obuhvaćena istraživanjem su sa iste farme, i u tom smislu, precizno poređenje majki sa ćerkama bilo je moguće, zbog zajedničkog uticaja farme i drugih faktora životne sredine u toku eksploatacije.

Ključne reči: krave, proizvodnja mleka, osobine mlečnosti, uvoz goveda

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Conflict of interest

The authors declare no conflict of interest.

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LARGE HERDS - GREATER CHALLENGES: COMPARATIVE ANALYSIS OF WELFARE QUALITY OF DAIRY COWS BY FARM SIZE

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Abstract: The aim of this study was to determine the effect of dairy herd size on the health score ("Good Health") and key welfare indicators of cows, using the *Welfare Quality*® methodology. Data were collected from small (≤ 100 cows), medium (101–300 cows), and large (> 300 cows) farms. More than 30 indicators were analyzed, including the prevalence of injuries, lameness, skin lesions, metabolic and reproductive disorders, mastitis, and mortality. The results showed that smaller-capacity farms achieved significantly higher ($p < 0.05$), health scores, lower prevalence of lameness, skin lesions, and metabolic disorders, as well as lower mortality rates compared with large farms. In large herds, higher incidences of mastitis, dystocia, and downer cow syndrome, along with increased mortality, were recorded, confirming the negative impact of production pressure and high animal aggregation on welfare. Mortality above the alarm threshold was occasionally observed on small and medium farms as well, indicating the necessity for continuous monitoring across all production systems. The findings suggest that optimizing herd size, improving housing conditions, and reducing production intensity are key strategies for achieving sustainable dairy production in line with animal welfare principles.

Key words: herd size, welfare quality, dairy cows, metabolic disorders, mortality, mastitis

Introduction

Although herd size has long been recognized as a relevant factor in dairy cow welfare, scientific evidence regarding this relationship remains fragmented. Previous research has predominantly focused on resource-based indicators, such as nutrition and housing, with earlier studies demonstrating that smaller herds tend to rank higher in these domains (Ostojić-Andrić et al., 2022). However, the impact of herd size on the health status of cows remains insufficiently elucidated, despite recent meta-analyses indicating a growing body of research on health indicators such as mastitis, laminitis, and biomarkers of metabolic stress (Linstädt et al., 2024; Wessels et al., 2025). These animal-based indicators reflect the direct response of the animal to its living conditions.

To date, most investigations have addressed this relationship through the prevalence of specific diseases and disorders (USDA, 2018; Ostojić-Andrić et al., 2022; Linstädt et al., 2024), with mixed results: certain studies report significant differences between small and large farms, while meta-analyses do not confirm a clear causal link (Wessels et al., 2025).

Among experts, there is a prevailing view that large-scale systems provide less favorable conditions for ensuring animal welfare. Increased farm capacity facilitates and accelerates pathogen spread, impedes active surveillance and timely detection of health issues, while intensive production further strains cow physiology (Lindena and Hess, 2022). Economically motivated space optimization-fewer resting places, limited feeders, and higher stocking density-often conflicts with high standards of welfare (Lindena and Hess, 2022; Popescu et al., 2007).

Globally, recent decades have seen a marked increase in herd size driven by consolidation in the dairy industry. In England, the average number of cows per farm rose by 27% between 2008 and 2018 (from 117 to 148; House of Commons Library, 2020), while in the EU-28 the increase was 69% (from 26 to 46; European Commission, 2021). Similar trends have been documented in Australia, New Zealand, and the United States, where current herd sizes are two to six times larger than in the 1990s (DairyNZ, 2014; Dairy Australia, 2015; MacDonald et al., 2007). This growth in capacity has coincided with wider adoption of modern technologies and reduced pasture access; by 2013, only about 25% of all dairy cows had access to pasture. Concurrently, selection and production pressures have intensified physiological stress and predisposition to production-related diseases (Coignard et al., 2014).

Contrary to global trends, the structure of cattle production in Serbia remains markedly small-scale. According to the 2023 Agricultural Census, 62% of cattle farms keep no more than nine cows, and the average household owns just one animal (RZS, 2024). Herds with more than 100 cows account for less than 1% of the total, yet contribute around 30% of milk sold (Ministry of Agriculture, 2025). This has resulted in a dual structure: small producers numerically dominate, while a limited number of large farms hold the leading market share. Large farms are characterized by “intensive” risks related to pathogens and metabolic stress, whereas smaller operations frequently face challenges of insufficient biosecurity and veterinary coverage (Agricultural Census, 2023).

While such changes serve to increase productivity, they also carry potential risks for dairy cow health that require timely management. Particular attention should be paid to monitoring metabolic disorders, which are highly prevalent and economically significant (EFSA, 2023). With a view to achieving more precise understanding of the relationship between herd size and welfare, this study examines the effect of farm capacity (≤ 100, 101–300, > 300 cows) on "Good Health" scores and key clinical indicators, testing the hypotheses H_0 (herd size has no effect) and H_1 (farms with > 300 cows have lower health scores and higher mortality).

Materials and Methods

Farms

The study encompassed a total of 16 conventional dairy farms, selected to ensure the greatest possible variability in herd size and housing system. The sample included farms operating under free-stall housing (FSH) and those using tie-stall housing (TSH) systems.

Based on the number of lactating cows, farms were classified into three categories: small (≤ 100 cows), medium (101–300 cows), and large (> 300 cows). In this paper, the terms herd size, farm capacity, and farm size are used interchangeably to denote the number of lactating cows on a farm, with the classification applied according to the aforementioned criteria.

The FSH system was present on 60% of large farms, 75% of medium farms, and 15% of small farms, while the remaining farms employed the TSH system. In terms of breed composition, 80% of the total cow population consisted of domestic Simmental, and the remaining 20% of Holstein-Friesian cattle.

To preserve the anonymity of participating farms and ensure clarity in data presentation, each farm was assigned a unique code from 1 to 16.

Welfare Assessment

Cow welfare was evaluated according to the Welfare Quality® Assessment Protocol for Cattle – WQP (2009), a standardized indicator system primarily based on animal-oriented measures that directly reflect the actual welfare status of the animals. On each farm, three trained assessors—experienced in dairy cow welfare assessment—conducted the evaluation. To eliminate the effects of seasonal variation, every farm was visited twice per year (in winter and summer), and the average value for each welfare measure was calculated.

Data collected from the farms were processed using the Welfare Quality® Scoring System (2023). More than 30 indicators—covering the domains of feeding, housing, health, and behavior—were aggregated into 12 criteria and four fundamental welfare principles (good feeding, good housing, good health, and appropriate behavior). According to the overall assessment score, farms were assigned to one of four welfare categories: excellent (81–100 points), enhanced (56–80 points), or acceptable (21–55 points). Farms failing to meet minimum requirements were classified as not classified (< 20 points). Since the aim of this study was to investigate the effect of herd size on the provision of adequate health status, Table 1 presents only the parameters included in the evaluation of this principle. A detailed description of each measure is provided in WQP (2009).

Statistical Analysis

All statistical analyses were performed using the commercial software Statistica v.10 (StatSoft, Inc., USA, 2015). For the evaluated measures, as well as for the scores of criteria and principles, descriptive statistical parameters were calculated, including the mean, standard deviation, and minimum and maximum values. The effect of herd size on welfare in the studied farms was assessed using the t-test or the Mann–Whitney test, depending on the normality of the data distribution as determined by the Kolmogorov–Smirnov test. Differences were considered statistically significant at $p < 0.05$.

Table 1. Criteria and measures used in the assessment of "Good health" principle (Welfare Quality® Assessment Protocol, 2009)

Principles	Criterions	Measures
Good health	Absence of injuries	Lameness; integument alterations
	Absence of disease	Coughing; nasal discharge; ocular discharge; hampered respiration; diarrhoea; vulvar discharge; milk somatic cell count; mortality; dystocia; downer cows
	Absence of pain induced by management procedure)	Disbudding/dehorning; tail docking

Results and Discussion

The results of the present study on the effect of farm size on the provision of the *Good Health* principle (Table 2) indicate that farm size exerted a significant influence ($p \leq 0.05$) on the health of dairy cows. The average health status across the farms surveyed fell within the *acceptable* welfare quality category (21–55 points), with the lowest score recorded on large-capacity farms (36 points).

Within the criterion *absence of injuries*, medium-capacity farms achieved the highest total scores, as well as the highest proportion of healthy cows and animals free from injuries. In contrast, large farms recorded the greatest proportion of moderately lame cows and those with skin lesions. These findings are consistent with earlier reports by Albana (1995) and Wells et al. (1999), as well as with more recent studies by Roche et al. (2024) and Patoliya et al. (2024), which indicate a four- to five-fold higher prevalence of hock lesions and a markedly greater incidence of lameness on farms with more than 300 cows compared to smaller systems. This may be attributed to increased stocking density, harder flooring, and reduced individual monitoring, which together limit lying comfort and delay the detection of early lesions (Barkema et al., 1999; Lindena and Hess, 2022).

Table 2. Comparison of welfare measures related to the “Good health”

Farm size	Large (over 300 cows)					Medium (100 - 300 cows)					Small (30 - 100 cows)					*F*	LSD test		
Number of farms, n	n=5					n=4					n=7						V	V	S
Principles, criterions and measures	$\bar{x}$	*SD*	S^2	*Min*	S	$\bar{x}$	*SD*	S^2	*Min*	*Max*	$\bar{x}$	*SD*	S^2	*Min*	*Max*		S	M	M
Good health	35.9	7.2	52.4	23.9	43.7	43.3	7.8	60.6	35.1	56.6	43.7	7.5	56.5	32.3	55.2	*	*	*	ns
1. Absence of injuries, points	46.3	16.3	267.0	21.0	67.9	58.7	12.4	153.9	45.1	81.1	51.3	14.3	204.9	32.8	81.1	ns	ns	ns	ns
Not lame cows, %	59.7	18.9	356.8	31.0	84.7	71.2	11.8	138.6	60.3	90.0	64.2	19.3	370.8	20.6	88.6	ns	ns	ns	ns
Moderately lame, %	31.0	13.6	183.6	12.5	49.5	24.9	10.2	104.2	9.2	35.9	24.3	15.6	243.6	7.0	61.8	ns	ns	ns	ns
Severely lame, %	9.3	6.7	44.6	2.2	20.3	4.0	2.6	6.9	0.5	8.2	16.1	21.0	441.1	0.0	86.6	ns	ns	ns	ns
Cows with at least one hairless patch, %	26.7	23.0	528.8	9.2	73.7	11.4	4.0	16.0	5.6	16.7	15.1	13.3	176.2	0.0	41.2	ns	*	ns	ns
Cows with at least one lesion, %	10.4	10.7	115.4	2.3	30.0	4.8	3.9	15.1	0.0	12.5	4.7	3.2	10.2	0.0	9.1	ns	ns	*	ns
Cows with no lesion, %	89.6	10.7	115.4	70.0	97.7	93.3	8.9	80.0	71.9	100.0	88.8	23.8	564.9	6.9	100.0	ns	ns	ns	ns
2. Absence of disease, points	51.2	14.4	207.2	30.2	64.6	54.4	20.0	399.5	40.4	100.0	68.4	24.6	607.3	36.7	100.0	ns	ns	ns	ns
Nasal	2.2	4.7	22.3	0.0	15.2	1.0	3.0	8.7	0.0	8.3	0.0	0.0	0.0	0.0	0.0	ns	ns	ns	ns

Farm size	Large (over 300 cows)					Medium (100 - 300 cows)					Small (30 - 100 cows)					*F*	LSD test		
Number of farms, n	n=5					n=4					n=7						V	V	S
Principles, criterions and measures	$\bar{x}$	*SD*	S^2	*Min*	S	$\bar{x}$	*SD*	S^2	*Min*	*Max*	$\bar{x}$	*SD*	S^2	*Min*	*Max*		S	M	M
discharge, %																			
Increased respiratory rate, %	0.2	0.4	0.2	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	ns	ns	*	ns
Ocular discharge, %	7.1	8.1	65.2	0.0	19.8	6.3	9.5	91.0	0.0	29.2	0.4	0.6	0.4	0.0	1.4	*	ns	*	ns
Diarrhoea, %	2.0	2.3	5.5	0.0	7.8	2.8	3.2	10.1	0.0	8.2	1.6	2.1	4.5	0.0	6.2	ns	ns	ns	ns
Vulvar discharge, %	0.9	0.9	0.9	0.0	2.5	1.1	0.9	0.8	0.0	2.5	2.0	1.9	3.8	0.0	5.6	ns	ns	ns	ns
Frequency of coughing, per cow per 15 min	0.2	0.4	0.2	0.0	1.0	0.1	0.4	0.1	0.0	1.0	0.0	0.0	0.0	0.0	0.0	ns	ns	ns	ns
Mastitis, %	2.6	1.4	2.0	1.6	5.3	2.0	1.0	0.9	0.7	3.5	2.2	0.7	0.5	0.8	3.4	ns	ns	ns	ns
Dystocia, %	2.9	2.1	4.3	0.8	6.2	8.1	8.2	67.3	2.2	21.3	2.9	4.1	17.0	0.0	9.7	*	*	ns	*
Downer cows, %	1.9	1.8	3.3	0.0	5.1	1.1	1.2	1.4	0.0	2.3	0.5	0.9	0.8	0.0	2.0	*	ns		ns
Mortality, %	10.7	7.1	50.5	1.3	21.3	4.4	2.3	5.2	1.1	6.3	5.2	5.2	27.1	0.0	16.7	*	*	*	ns
3. Absence of pain induced by management, points	28.0	0.0	0.0	28.0	28.0	46.0	33.3	1110.9	28.0	100.0	47.4	34.6	1198.4	20.0	100.0	ns	ns	ns	ns
Disbudding/ dehorning, %	100.0	0.0	0.0	100.0	100.0	75.0	46.3	2142.9	0.0	100.0	65.6	44.2	1953.9	0.0	100.0	ns	ns	*	ns

p = significance of differences between the means calculated by t-test, ns = not significant (p> 0.05), * = significant statistical differences at p< 0.05, ** = significant statistical differences at p< 0.01

The criterion *absence of disease* was rated most favorably on small farms, whereas the poorest results were documented on large farms. In the latter, a higher proportion of metabolic disorders was recorded, in line with the findings of Linstädt et al. (2024), which confirm that the prevalence of metabolic disorders and mastitis increases proportionally with herd size. This can largely be explained by the higher production intensity and negative energy balance typical of large-capacity farms, which place additional physiological strain on high-yielding cows during early lactation and increase their susceptibility to metabolic disturbances (Egger-Danner et al., 2020; EFSA, 2023). Metabolic disorders represent key risk factors that impair health scores and prolong the service period of cows, and are often subclinical-such as subclinical hypocalcaemia (SCHC), whose prevalence on large farms reaches 35-50% (Berean et al., 2025). Higher stocking densities in such systems facilitate the spread of infectious agents and hinder timely diagnosis (Mramba and Mohamed, 2024), while greater production intensity further strains physiological functions and reduces the capacity to maintain welfare (Egger-Danner et al., 2020; EFSA, 2023). Economically driven infrastructural solutions—such as a reduced number of stalls and feeding places combined with higher stocking density—may increase efficiency but negatively affect welfare (Steeneveld et al., 2024). The association between reduced comfort and higher incidence of mastitis and laminitis has also been confirmed in earlier research (Barkema et al., 1999; Lindena and Hess, 2022).

Diseases such as coughing, downer cow syndrome, nasal and ocular discharge, tachypnoea, and mastitis were more prevalent on large farms, in accordance with the findings of Waage et al. (1998). Such patterns are likely a consequence of higher animal aggregation, which facilitates faster pathogen transmission, combined with suboptimal ventilation and delayed disease detection in large groups (Lindena and Hess, 2022; EFSA, 2023). On medium-capacity farms, a high incidence of dystocia (8.14%) was observed, notably exceeding the alarm threshold of 5.5% (Forkman and Keeling, 2009). The elevated incidence of dystocia and downer cow syndrome in medium and large herds reflects the combined effects of genetic selection for milk yield and the associated reproductive and metabolic challenges experienced by high-producing cows (EFSA, 2023; Steeneveld et al., 2024). These findings further support the EFSA (2023) conclusion that large dairy herds are a reliable predictor of increased risk of mastitis and other production-related diseases.

The highest mortality rate (10.71%) was recorded on farms with more than 300 cows; however, small (5.16%) and medium farms (4.37%) also exceeded the alarm threshold (Forkman and Keeling, 2009). A pilot study conducted on 12 pasture-based farms in Uruguay reported an average mortality rate of 4.5% (range 1.1–8.1%), with values above 8% considered a serious welfare concern (Doncel-Díaz et al., 2025).

In addition, the routine practice of dehorning, performed consistently and without anaesthesia on large farms (100% of animals), resulted in lower scores for the criterion absence of pain induced by management procedures. On smaller farms, this practice was carried out less frequently, in approximately two-thirds of cows.

Conclusion

The findings of this study confirm that herd size exerts a significant influence on the health status of dairy cows, with large-capacity farms (> 300 cows) achieving lower scores within the Good Health principle compared with smaller production systems. Higher rates of metabolic disorders, mastitis, dystocia, injuries, and mortality were recorded on larger farms, indicating a complex interaction between production intensity, biosecurity challenges, and the physiological burden placed on animals. In contrast, small and medium-sized farms generally achieved more favorable results for most indicators, although they were not entirely free from critical values for certain parameters.

These results suggest that, while increasing farm capacity may improve economic efficiency, it entails substantial risks to cow welfare and health-particularly with respect to metabolic and infectious diseases. Therefore, the expansion of production on large farms should be accompanied by systematic monitoring of animal-based indicators, enhancement of biosecurity measures, optimization of housing infrastructure, and adjustment of production intensity to align with the physiological capacities of the animals. Special emphasis should be placed on the early detection and prevention of metabolic disorders, the provision of adequate lying and feeding space, and the humane execution of routine procedures such as dehorning.

Integrating these measures into farm management practices could contribute to reducing health risks and improving overall welfare standards, thereby ensuring the sustainability of dairy production in Serbia in line with contemporary standards and consumer expectations.

Veća stada – veći izazovi: komparativna analiza kvaliteta dobrobiti mlečnih krava u zavisnosti od veličine farme

Dušica Ostojić Andrić, Ljiljana Samolovac, Dragan Nikšić, Miloš Marinković, Nenad Mićić, Danijel Milenković, Dobrila Janković

Rezime

Cilj ovog istraživanja bio je da se utvrdi uticaj veličine mlečnog stada na zdravstveni skor (princip Dobro zdravlje) i ključne indikatore dobrobiti krava, koristeći metodologiju Welfare Quality®. Podaci su prikupljeni na malim (≤ 100 grla), srednjim (101–300) i velikim (> 300) farmama. Analizirano je više od 30 indikatora, uključujući učestalost povreda, šepavosti, kožnih lezija, metaboličkih i reproduktivnih poremećaja, mastitisa i mortaliteta. Rezultati su pokazali da farme

manjeg kapaciteta ostvaruju značajno više ($p < 0.05$) ocene zdravstvenog skora, manju prevalencu šepavosti, lezija i metaboličkih poremećaja, niži mortalitet, u poređenju sa velikim farmama. Na velikim farmama registrovana je veća učestalost mastitisa, distokija i sindroma ležanja, kao i viši mortalitet, što potvrđuje negativan uticaj proizvodnog pritiska i visoke aglomeracije životinja na njihovu dobrobit. Mortalitet iznad alarmantnog praga povremeno je registrovan i na manjim i srednjim farmama, što ukazuje na potrebu stalnog monitoringa u svim proizvodnim sistemima. Rezultati sugerišu da optimizacija veličine stada, unapređenje uslova držanja i smanjenje intenziteta proizvodnje predstavljaju ključne smernice za postizanje održive mlečne proizvodnje u skladu sa principima dobrobiti životinja.

Ključne reči: veličina stada, kvalitet dobrobiti, mlečne krave, metabolički poremećaji, mortalitet, mastitis

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

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SEASONAL VARIATIONS IN EGG QUALITY TRAITS IN BELGRADE SUPERMARKETS

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Abstract: The aim of this study was to determine the quality of eggs available in Belgrade supermarkets during different seasons (winter, spring, summer), for various housing systems (cage, floor, and free-range), and to assess the representation of eggs from cage and non-cage systems in the year when Serbian egg producers have been granted an extended transitional period to comply with animal welfare legislation banning conventional cage systems. The research covered nine Belgrade supermarkets. All egg producers representing the three housing systems were recorded, and eggs up to 15 days old were tested for external and internal quality traits. The results show that cage-system producers remain dominant on the market, with a share ranging from 45.5% in winter to 50% in spring and summer. The share of floor (non-cage) eggs was 36.6% in winter and 30% in spring and summer, while free-range eggs had the lowest representation during all three seasons (around 20%). The findings indicate variability in egg quality traits influenced by both season and housing system as the second studied factor. Haugh Unit (HU) values, which represent an aggregate indicator of egg quality, were highest in winter, averaging 76.29 for the cage system, 76.31 for the floor system, and 77.28 for the free-range system. In spring and summer, lower HU values were recorded for all three systems, with a more pronounced decline in the non-cage systems. It can be concluded that there is a growing trend in the supply of eggs from non-cage systems in Belgrade supermarkets, which is more favorable in terms of animal welfare. The development of these systems could be further supported through education of producers and consumers, as well as through research and producer incentives. Although the study focuses on egg quality at the market level, and thus reflects the quality of the product reaching consumers, it also indicates potential weaknesses in the “from producer to consumer” chain. Therefore, it can be a starting point for future research on the entire system of egg production and distribution.

Key words: egg quality, season, housing systems, retail – supermarkets

Introduction

Table eggs are an important component of human diets worldwide due to their affordable price and high nutritional value, which makes them an ideal food for the daily diet of people of all age groups (Tolimir et al., 2017; Rafed et al., 2024). However, when analyzing data on egg consumption in Serbia, which amounted to 217 eggs per capita in 2023 (Statistical Office of the Republic of Serbia, Household Budget Survey, 2023), it can be seen that this figure is significantly lower compared to countries such as Mexico (368), Japan (337), China (255), Spain (273), and Denmark (248) (World Egg Organisation, 2018). One of the reasons for the lower egg consumption may be consumers’ concerns related to “cholesterophobia”, and therefore, education of consumers is essential. In this context, studies focusing on the nutritional value of eggs with an emphasis on the health aspect are particularly important, as they point out that cholesterophobia-the fear of consuming eggs due to potential health risks, most commonly cardiovascular diseases-is ungrounded. The health aspect has been the subject of numerous studies that generally summarize the key nutritional properties of eggs within a balanced diet and highlight their benefits for human health (Réhault-Godbert et al., 2019; Pal and Molnar, 2021; Virtanen and Larsson, 2024). According to Myers and Ruxton (2023), in European countries, an increased egg consumption would contribute to health benefits of the population.

In addition to the chemical composition and nutritional value of eggs, an important area of research is egg quality, especially from the perspective of food safety, different production systems, and consumer protection. The quality traits of eggs (external and internal) are influenced by factors prior to laying - such as genetics, hen age, nutrition, and housing system - as well as by factors after laying, including egg handling, i.e. packaging technology, transport, and storage conditions. Research in this field has been extensive and often involves the simultaneous examination of multiple factors (Feddern et al., 2017; Kopacz and Drażbo, 2018; Tavares et al., 2018; Polat Yurtseven et al., 2021; Rakonjac et al., 2021; Škrbić et al., 2021; Kim et al., 2022). From the consumer perspective, the most important aspect is the quality of eggs on the retail market, which serves as an indicator of the efficiency of the entire production and distribution chain. Pavlovski et al. (2007) emphasize that eggs produced on the farm may have good initial quality, which can be compromised by improper handling and storage conditions both on the farm and throughout the marketing channels. Therefore, studies on egg quality in retail (Jones and Musgrove, 2005; Khan et al., 2013) are particularly important, as their goal is to determine the external and internal quality traits of eggs under the influence of different storage conditions and egg handling. Apart from physical traits, egg quality assessments on the market include the monitoring of chemical (Petrovska et al., 2018) and microbiological changes in eggs (Omkarappa et al., 2019), as well as the assessment of variations in the nutritional quality of eggs in retail, depending on the genotype, housing system, and season (Akyurek and Okur, 2009; Feddern et al., 2017; Alshaikhi et al., 2021; Chatzidimitriou et al., 2024).

There are relatively few data available in Serbia on the quality of eggs in supermarkets, and even less on how quality of these eggs varies depending on the season and housing system. Therefore, the aim of this study was to examine egg quality traits across three seasons (winter, spring, and summer), as well as to highlight potential differences among housing systems (cage, floor, and free-range) under specific market conditions. Additionally, the study aimed to assess how the process of complying with animal welfare regulations, including the ban on conventional cages for laying hens, affected the supply of eggs from alternative production systems in the Belgrade market.

Material and Methods

The study on the supply and quality of table eggs from three production systems-cage, floor, and free-range-across three different seasons (winter, spring, and summer 2025) was conducted in the territory of Belgrade, in nine retail outlets-supermarkets. The egg supply was determined by recording all producers represented in each supermarket, with the aim of identifying the seasonal dynamics in the supply of eggs from different systems. Egg quality assessment was performed on samples from all supermarkets, with 10 medium-sized (class M) eggs per producer, aged up to 15 days (the number of days from the packaging to the purchase did not exceed 15 days), to eliminate the effect of age on egg quality traits and to ensure conditions for a valid and mutually comparable assessment of egg quality. The sampling of all eggs was done in a single day, and quality parameters were evaluated the following day, during which the eggs were stored in a refrigerator. For each egg, the researchers analyzed external traits (egg weight, cleanliness and shell color, and egg shape index) and internal quality traits (albumen height, Haugh units, yolk color, and shell thickness). All analyses were carried out by using standard assessment methods: egg weight was determined by using an electronic scale with ±0.01 g accuracy; shell cleanliness and color were assessed visually, on a scale from 1 (dirtiest/lightest) to 5 (cleanest/darkest). Egg shape index was calculated as the ratio of width (mm) to length (mm) of the egg, measured by using a template for determining egg length and width. Albumen height was measured at the midpoint between the yolk edge and the thick albumen layer by using an AMES tripod micrometer with 0.1 mm precision. Yolk colour was assessed visually, comparing with the Roche Yolk Color fan (scale 1–15 for color intensity). Shell thickness was measured with a micrometer and expressed in µm. Haugh units (HU) were calculated using the following formula: $HU = 100 \cdot \log10 (H - 1.7 \cdot W^{0,37} + 7.6)$, where H is the albumen height (mm), and W is the egg weight (g). The recorded average monthly temperatures for Belgrade were 3.3°C in February, 16.8°C in May, and 25.4°C in July, as retrieved from monthly bulletins issued by the National Hydrometeorological Service of Serbia (RHMZ, 2025). The obtained results were processed with the statistical software package STATISTICA (Statistical), ANOVA procedure. The significance of the differences was assessed at the probability level of $p \leq 0.05$ by Tukey test.

Results and Discussion

Table 1 presents the results of the survey on the egg supply in Belgrade supermarkets in the winter, spring, and summer seasons of 2025 from different production systems-cage, floor, and free-range.

Table 1. Seasonal variations in egg supply in Belgrade supermarkets given by different housing systems

Housing system	**SM1**	**SM2**	**SM3**	**SM4**	**SM5**	**SM6**	**SM7**	**SM8**	**SM9**	**Total supply 1**
					Winter 2025					
Cage	P2	P3	P4	P3	P5	P3	-	P6	P3	5
Floor	P1, P2	P3	P4	P3	-	-	P3	-	-	4
Free range	P2	-	-	P3	-	-	P3	-	P2	2
Total supply 2	2	1	1	1	1	1	1	1	2	
					Spring 2025					
Cage	P7	P3	P4	-	-	P8	0	P6	-	5
Floor	P1, P2	P3	0	-	-	-	0	-	P2	3
Free range	P2	P3	P2	-	-	-	P3	-	P2	2
Total supply 2	3	1	2			1	1	1	1	
					Summer 2025					
Cage	P1	P3	P1, P4	P3	P8	-	-	P8	P6	5
Floor	P1, P2	P1	P3	P3	-	P3	P3	-	P2	3
Free range	P2	P3	-	P3	P2	-	P3	-	P2	2
Total supply 2	2	2	3	1	2	1	1	1	2	

SM – abbreviation for supermarket; P – abbreviation for the producer
Total supply 1 - number of producers by housing system
Total supply 2 - number of producers by supermarket

Based on the data in Table 1, it can be seen that the supply of eggs from different production systems (cage, floor, and free-range) in the winter, spring, and summer periods was relatively stable, ranging from 10 different brands in the spring and summer seasons to 11 in the winter, considering all supermarkets and production systems together. When comparing the cage system with the floor and free-range systems, it was found that the supply of eggs from cage systems was predominant throughout all three seasons. However, when comparing the cage system with non-cage systems (floor and free-range combined), it can be concluded that the cage system was slightly dominant only during the winter, with 55% of the total supply, while in spring and summer this ratio equalized (50:50). This may indicate a market transition influenced by legislative changes. Analyzing the supply from the producers' perspective, it can be concluded that only two producers (P2 and P3) were represented with eggs from multiple production systems across several seasons, suggesting that only a small number of producers have the flexibility and capacity to adapt to the increasingly demanding market through diversification of production. Most producers remain focused on more economical forms of production, while the availability of more ethically acceptable options floor and free-range eggs remains limited. The obtained results confirm that the transition toward more sustainable production systems has been progressing slowly, with a slight upward trend, which has also been confirmed in similar previous studies (Tolimir et al., 2020; 2024). Sinclair et al. (2022) pointed out that consumer attitudes and knowledge regarding eggs outside EU countries were not entirely understood. They conducted a survey in 14 non-EU countries, showing that consumers' understanding of egg production systems varied greatly, often with lack of knowledge, and regional differences both in the level of awareness and attention paid to animal welfare. Analyzing the situation globally, the authors stated that conventional cages were first banned in Europe in 2012, after which that the process of phasing out conventional caged egg production started in Canada, Australia, and New Zealand. However, they emphasized that the majority of eggs worldwide were still produced in cage systems, despite significant public awareness campaigns on animal welfare conducted in some countries. Rahmani et al. (2019) and Doyon et al. (2023) highlighted the importance of consumer awareness and their preferences toward eggs from different housing systems, considering animal welfare and environmental impact. Zinca et al. (2024) emphasized the effects of various housing systems on egg quality and pointed out that these systems remained a controversial topic among consumers, producers, researchers, and environmentalists, particularly regarding hen health, behavior, and egg quality. In Serbia, the transition from conventional cage systems to permitted (alternative) systems is directly influenced by legal regulations, according to which the final deadline has been re-extended (Rulebook on Conditions for Animal Welfare, 2024). Radulović et al. (2025) analysed the egg production sector and identified insufficient compliance with welfare standards as one of its weaknesses.

Considering that consumer attitudes can also influence a shift toward non-cage systems, education and better public awareness about production systems and egg quality would be of great importance in Serbia.

Table 2 presents data on the quality of eggs from different production systems - cage, floor, and free-range, in three seasons - winter, spring, and summer – in Belgrade supermarkets. According to the results, the season has a significant impact on both internal and external egg quality traits. Haugh units (HU), as the overall and most important indicator of egg freshness, showed the smallest seasonal variations in the cage system throughout all three seasons, indicating greater stability of the egg quality compared to the floor and free-range systems. However, although the results suggest that the cage production system is characterized by the smallest seasonal fluctuations in Haugh units, it should be considered that the research was conducted on eggs obtained from supermarkets. This leads to the question whether the observed stability in egg quality in the cage system is a result of the production system itself or of differences in handling, storage, and transportation of eggs to the retail location.

Also, based on the obtained results, it can be observed that Haugh unit (HU) values were the highest during winter for all three systems, while in warmer seasons, a decline in values occurred, which was more pronounced in eggs from floor and free-range systems. The finding that a decrease in certain egg quality parameters was recorded for all three systems indicates that none of the systems is completely immune to seasonal stress. The fact that retail eggs had higher HU values during winter was also confirmed by the study of Alshaikhi et al. (2021). The obtained results can be linked to higher temperatures during spring and summer, when several factors combine - accelerated biochemical changes due to higher temperatures, more difficult and slower cooling of eggs immediately after laying, as well as a potentially less efficient cold chain during storage and transportation to retail outlets in spring and summer. The negative effect of prolonged storage time and temperature on HU was also confirmed by Akyrek and Okur (2009) and Škrbić et al. (2021).

The obtained results can be interpreted in relation to the study by Feddern et al. (2017), who identified ambient temperature and relative humidity as key factors that negatively affect egg quality traits. Al-Obaidi and Al-Shadeedi (2018) reported greater variation in certain egg quality characteristics during the summer compared to the winter period. The importance and influence of the post-laying period on egg quality were highlighted by Kim et al. (2022), who also pointed out the challenge of maintaining egg quality during storage and transportation due to changes in external factors such as temperature and humidity. They emphasized the need to develop models for estimating Haugh units based on data on weight loss under cold chain conditions.

Table 2. Seasonal variations of egg quality in Belgrade supermarkets, by housing systems

Season	Sistem		Egg weight	Shell cleanliness	Shell colour	Egg shape index	Yolk colour	HU	Shell thickness
Winter	Cage	$\bar{X}$	59.29^{c}	4.78^{ab}	3.85^{de}	82.43^{ns}	12.34^{a}	76.29^{d}	38.97^{abc}
		Sd	±2.57	±0.78	±0.63	±3.36	±0.60	±8.24	±2.75
Spring		$\bar{X}$	58.63^{bc}	4.73^{ab}	3.69^{cd}	83.80^{ns}	12.57^{ab}	71.18^{bcd}	39.94^{c}
		Sd	±2.37	±0.61	±0.58	±3,02	±1.23	±13.27	±2.82
Summer		$\bar{X}$	57.37^{ab}	4.31^{a}	3.34^{abc}	81.67^{ns}	12.39^{a}	71.94^{cd}	37.91^{a}
		Sd	±2.68	±1.28	±0.67	±8.44	±1.52	±8.14	±2.88
Winter	Floor	$\bar{X}$	57.54^{abc}	4.66^{ab}	3.80^{de}	83.06^{ns}	12.39^{a}	76.31^{d}	37.76^{a}
		Sd	±2.53	±1.00	±0.62	±3.81	±0.67	±10.71	±2.67
Spring		$\bar{X}$	59.36^{c}	4.62^{ab}	3.90^{de}	82.73^{ns}	12.56^{a}	65.68^{ab}	39.15^{abc}
		Sd	2.53	1.05	0.54	2.78	0.81	9.76	±1.59
Summer		$\bar{X}$	56.88^{a}	4.42^{ab}	3.27^{a}	81.82^{ns}	12.50^{a}	67.94^{abc}	37.94^{a}
		Sd	±3.34	±1.25	±0.72	±2.56	±1.42	±11.65	±2.79
Winter	Free- range	$\bar{X}$	59.45^{c}	5.00^{b}	4.22^{e}	82.82^{ns}	12.28^{a}	77.28^{d}	39.61^{bc}
		Sd	±2.83	±0.00	±0.63	±3.71	±0.66	±6.19	±2.48
Spring		$\bar{X}$	58.73^{bc}	4.28^{a}	3.70^{bcd}	82.80^{ns}	13.14^{b}	64.80^{ab}	39.14^{abc}
		Sd	±1.89	±1.44	±0.68	±3.74	±0.74	±10.89	±2.29
Summer		$\bar{X}$	57.27^{ab}	4.42^{ab}	3.24^{a}	80.87^{ns}	12.16^{a}	63.41^{a}	38.18^{ab}
		Sd	±2.53	±0.98	±0.59	±2.54	±0.83	±10.44	±2.31
p-value			0.000	0.014	0.000	0.120	0.001	0.000	0.000

$^{a-e}$ - Different letters indicate significant differences among the means in each column
^{ns}- not statistically significant differences among the means in the column

In addition to analyzing the Haugh unit (HU) as an overall indicator of egg quality, other egg traits presented in Table 2 can be discussed. Eggshell thickness was the lowest during the summer in all three housing systems, which may be attributed to the seasonal influence on shell mineralization. Conversely, the highest shell thickness was observed in spring, which is consistent with the findings of Ikusika et al. (2025), who explained this result by the improved calcium metabolism due to lower thermal stress in spring compared with the winter period. The obtained results for HU and shell thickness indicate that special attention should be paid to eggs collected in the summer period because of the potentially higher risk of shell breakage and reduced quality preservation. Yolk colour remained relatively consistent across the three seasons, with the highest values observed in free-range eggs during spring, which can be linked to the more diverse diet of hens and their access to green pastures. This is in agreement with previous findings (Hammershøj and Johansen, 2016; Gumulka et al., 2017).

These observations confirm that, both season and the housing system significantly affect the nutritional and physical properties of eggs, which should be taken into account when assessing the market quality of eggs.

Figure 1 presents the egg quality in Belgrade supermarkets, expressed as HU, in different seasons, by producers and housing systems.

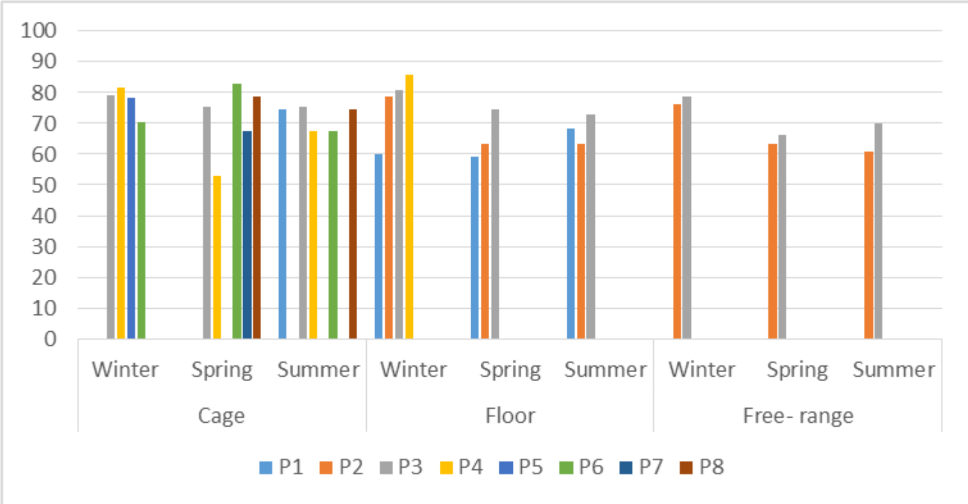

Figure 1. Egg quality in belgrade supermarkets, expressed in HU in different seasons by producers and housing systems

The results indicate that the quality of eggs available on the market shows pronounced variations in Haugh units depending on the season, with the highest quality recorded during the winter period, while a noticeable decline was observed in summer, particularly in eggs from free-range and floor systems. According to the study by Freitas et al. (2023), the variability of egg quality traits is greater in the summer than in the winter season, while the influence of both season and housing system on egg quality was also confirmed by Rakonjac et al. (2018). The question of whether the obtained retail egg quality is a result of the factors before laying - different genotypes, nutrition, housing conditions, hens' age and health or is a consequence of factors influencing egg quality after laying – temperature, relative humidity, presence of dust, foreign odors, and other effects during storage and transport - highlights the need for more extensive research covering the entire chain from the farm to retail, i.e., to the consumer. According to Roberts et al. (2004), a systematic improvement of egg quality would involve limiting the age of hens from which eggs are sourced, shortening the storage period before processing and distribution, enriching the hens' diet with the vitamin-mineral supplements and establishing higher grading standards. When observed by producer, HU values were the highest during the winter period for three producers (P2, P3, and P4) across all three housing systems. However, it can be noted that for certain producers (P1 and P6), the variation in HU could not be linked to seasonal trends, as no consistent pattern was observed. Due to the instability of supply and the lack of continuous seasonal availability, some producers (P5 and P7) could not be analyzed by season. It is important to emphasize that the study was conducted on samples collected from retail outlets; therefore, the differences observed among producers cannot be interpreted solely through the lens of production factors, but must also take into account transport conditions, storage duration, and storage regimes in supermarkets, which are particularly critical during warmer months. In this regard, the observed variations in egg quality reflect the complexity of the supply chain rather than production technology alone, highlighting the importance of maintaining quality control throughout the entire distribution pathway - from farm to consumer.

Conclusion

In line with the objective of this study, and based on the obtained results, several conclusions were drawn. The findings indicate a gradual transition toward non-cage systems, while eggs from cage systems remain dominant—both in terms of the number of producers represented in supermarkets and the range of products offered within each Belgrade supermarket. The further transition to non-cage systems in Serbia is directly influenced by legislative decisions regarding the definition or extension of deadlines for the ban on conventional cages. Additionally, this process could be affected by producer and consumer education, as well as by incentives for producers investing in permitted, alternative, and more humane housing systems. The results of the egg quality assessment in supermarkets indicate variability of traits under the influence of season, with more pronounced variations observed in eggs from alternative production systems (floor and free-range) compared with the cage system. Less favorable Haugh unit (HU) values, as an overall indicator of egg quality, during warmer seasons point to the need for special attention to preserving egg quality during these periods through the optimization of storage and transport conditions, as well as through the development of a systematic approach to monitoring quality under market conditions. Based on the obtained results, it is recommended to conduct further research encompassing the entire egg production chain-from farm to retail point-in order to identify the causes of variations in quality.

Sezonske varijacije u karakteristikama kvaliteta jaja u beogradskim supermarketima

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Rezime

Cilj rada bio je da se utvrdi kvalitet jaja u beogradskim supermarketima tokom različitih sezona (zima, proleće, leto), za različite sisteme gajenja (kavezni, podni i slobodni) i da se proceni zastupljenost jaja iz kaveznih i nekaveznih sistema u periodu ponovnog produženja roka za usklađivanje sa zakonskim propisima o dobrobiti životinja kojima se zabranjuju konvencionalni kavezni sistemi. Istraživanje je obuhvatilo devet beogradskih supermarketa. Svi proizvođači jaja iz sva tri sistema su evidentirani, a jaj a starosti do 15 dana su uzorkovana i testirana na spoljašnje i unutrašnje osobine kvaliteta. Rezultati pokazuju da su proizvođači iz kaveznih sistema dominantni na tržištu, sa udelom u rasponu od 45,5% zimi do 50% u proleće i leto. Udeo jaja iz podnog (nekaveznog) držanja bio je 36,6% zimi i 30% u proleće i leto, dok su jaja iz slobodnog uzgoja imala najmanju zastupljenost tokom sva tri sezone (oko 20%). Rezultati ukazuju na varijabilnost osobina kvaliteta jaja pod uticajem sezone i sistema gajenja, kao drugog proučavanog faktora. Vrednosti Haugovih jedinica (HJ), koje predstavljaju zbirni indikator kvaliteta jaja, bile su najviše zimi, u proseku 76,29 za kavezni sistem, 76,31 za podni sistem i 77,28 za sistem slobodnog gajenja. U proleće i leto zabeležene su niže vrednosti HJ za sva tri sistema, sa izraženijim padom u nekaveznim sistemima. Može se zaključiti da postoji rastući trend u ponudi jaja iz nekaveznih sistema u beogradskim supermarketima, što je povoljnije sa stanovišta dobrobiti životinja. Razvoj ovih sistema mogao bi se dodatno podržati kroz edukaciju proizvođača i potrošača, kao i kroz istraživanje i podsticaje proizvođačima. Iako se rad fokusira na kvalitet jaja na nivou tržišta i time odražava kvalitet proizvoda koji stiže do potrošača, on takođe ukazuje na potencijalne slabosti u lancu „od proizvođača do potrošača“. Stoga rad može biti polazna tačka za buduća istraživanja celog sistema proizvodnje i distribucije jaja.

Ključne reči: kvalitet jaja, sezona, sistemi gajenja, maloprodaja – supermarketi

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Conflict of interest

The authors declare no conflict of interest.

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